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THE METABOLISM OF l-XYLOSE

By HARDY W. LARSON, N. R. BLATHERWICK, PHOEBE J. BRADSHAW, MARY E. EWING, and SUSAN D. SAWYER

(From the Biochemical Laboratory of the Metropolitan Life Insurance Company, New York)

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Miller and Lewis (1) studied the metabolism of *d*-xylose in the rat and found it to be non-glycogenic and poorly absorbed. Increased pentose content of the liver, kidney, and blood resulted after its administration. Blatherwick *et al.* (2) reported no increase in glycogen content of liver and muscle after *d*-xylose was fed to rats, and no change in the lactic acid content of liver, muscle, and blood was observed. There was a rise in the non-fermentable reducing substances of the liver, muscle, kidney, and blood and a significant increase in blood glucose. Miller and Lewis also noted an increase in the fermentable reducing substances of the blood but questioned its significance. Blanco (3) observed a similar increase in blood glucose after the administration of *d*-xylose to rabbits. Marble and Strieck (4) found a slight rise in the respiratory quotient of normal and diabetic men and normal and phlorhizinized dogs after the ingestion of *d*-xylose and concluded that oxidation of the sugar had taken place. Magendantz (5) fed *d*-xylose to dogs which had fasted for 2 or 3 weeks and found that it caused considerable diuresis and a marked diminution of nitrogen excretion, indicating that the carbohydrate exerted a protein-sparing action. However, he obtained no evidence that it induced glycogen formation even when fed in large amounts. According to Nothdurft (6) *d*-xylose was partially utilized by man and guinea pig.

Although considerable work has been done on the utilization of this pentose, its optical isomer, *l*-xylose, has received but scant attention and apparently no study of its metabolism has been undertaken. This sugar is of particular interest because of its

close relationship to the ketopentose, *l*-xylulose, which occurs in pentosuria, and also because of its use in the synthesis of ascorbic acid. The present paper deals with the question of the utilization of this sugar by the animal organism.

Preparation of l-Xylose—*l*-Xylose was synthesized from *d*-sorbitol by the method of von Vargha (7). 310 gm. of 80 per cent sorbitol syrup[1] were shaken with 100 cc. of water, 135 gm. of benzaldehyde, and 25 cc. of concentrated HCl for 6 hours, after which time most of the monobenzal sorbitol had crystallized. The crystals were filtered by suction before any of the amorphous dibenzal sorbitol had precipitated. They were washed with ice water, then removed from the filter and placed in a mortar and thoroughly mixed with ice water, and again filtered. The crystals were then ground with sufficient alcohol to make a thin paste, and filtered. The monobenzal sorbitol was washed with a small amount of ether and dried at 50°. This treatment was found to produce a sufficiently pure compound (m.p. 171°) without further recrystallization.

For the preparation of monobenzal *l*-xylofuranose, 85 gm. of monobenzal sorbitol were shaken with 140 gm. of $Pb(C_2H_3O_2)_4$ in 750 cc. of glacial acetic acid for 1 hour, after which the acid was distilled off *in vacuo* at 40°.[2] The residual syrup was dissolved in 300 cc. of cold water, and extracted with 930 cc. of C.P. ethyl acetate. If the ethyl acetate was not of the highest purity, extraction was not complete and an impure product resulted. The ester solution was washed twice with 300 cc. portions of water to

[1] The sorbitol syrup used in the synthesis of *l*-xylose was generously furnished by the Atlas Powder Company.

[2] For the preparation of lead tetraacetate (8) 500 cc. of glacial acetic acid were heated to 50-60°. 200 gm. of Pb_3O_4 were added in small portions and the mixture stirred mechanically after each addition until the red color disappeared. Toward the end of the reaction a small amount of undissolved lead tetraoxide remained. The lead tetraacetate was allowed to crystallize overnight in the refrigerator in a tightly stoppered bottle, then filtered on a Buchner funnel, and washed with a small amount of cold glacial acetic acid. Yield 150 gm. If suction was continued too long, the tetraacetate decomposed. Recrystallization was found unnecessary for the above synthesis. The moist crystals were quickly transferred to a shaking bottle, weighed, and the appropriate amounts of glacial acetic acid and monobenzal sorbitol added before decomposition of the lead tetraacetate could take place.

remove any trace of lead. A small amount of water was then added and the extract evaporated to a thick syrup *in vacuo*. 60 gm. of the syrup were refluxed on the water bath for 1 hour with 600 cc. of 10 per cent acetic acid. The acid and liberated benzaldehyde were distilled *in vacuo* at 40°. The *l*-xylose syrup was dissolved in water, treated with norit, and concentrated *in vacuo* several times. The thick syrup was then dissolved in hot ethyl alcohol, and, after it had cooled, ether was added to a slight turbidity. Crystallization took place readily. Yield about 25 gm. $[\alpha]_D^{20} = -18.6° \pm 0.2°$.

EXPERIMENTAL

A group of normal adult white rats (aged 95 to 119 days) from our stock colony and previously fed our stock diet was used for the experiment. They were fasted 24 hours prior to the feeding of the *l*-xylose. 2 cc. of a 25 per cent solution of the sugar were then given by stomach tube and after a 3 hour absorption period the rats were anesthetized with sodium amytal; the hind legs were then skinned, and after being frozen *in situ* with CO_2 snow the legs and liver were removed and placed in liquid air. The technique employed for the preparation of the tissues for analysis and the methods used for the determination of glycogen, lactic acid, fermentable and non-fermentable sugar have been described in an earlier paper (9).

A summary of the results obtained with the experimental animals together with those for the 27 hour control rats is given in Table I. The control values were obtained over a period of 4 years in other experiments dealing with carbohydrate utilization, and were sufficiently uniform to justify combining them. As the average liver glycogen content of fasting males remains at a considerably higher level than that of females, the hepatic glycogen values have been grouped according to the sex of the animal. There was no apparent sex difference in muscle glycogen values.

The results of the tissue analyses are expressed as maximal, minimal, mean, and standard deviation of the mean values.

From an inspection of these results it is apparent that *l*-xylose is not utilized by the rat. No marked increases were noted in the glycogen, lactic acid, and fermentable reducing substances after

TABLE I

Carbohydrate Content of Tissues of Control and Experimental Rats

The results are expressed in mg. per 100 gm., except for blood in which case they are recorded as mg. per 100 cc. The values for glycogen are given in terms of glucose. The figures in bold-faced type represent values for the rats given *l*-xylose.

Substance determined	Maximal	Minimal	Mean	Standard deviation of mean	No. of rats	
					Males	Females
Glycogen, liver	603	26	203	28		30
" "	**467**	**58**	**216**	**42**		**8**
" "	1847	94	535	67	41	
" "	**609**	**75**	**273**	**46**	**10**	
" muscle	749	370	563	11	40	29
" "	**574**	**422**	**509***	**10**	**10**	**8**
Lactic acid, liver	17.5	5.5	10.0	0.5	23	14
" " "	**10.8**	**5.5**	**7.3***	**0.4**	**10**	**8**
" " muscle	54.2	11.8	22.8	1.2	31	20
" " "	**52.0**	**13.6**	**24.3**	**2.1**	**10**	**8**
" " blood	21.8	8.1	13.8	1.0	8	8
" " "	**15.8**	**6.8**	**10.6***	**1.0**	**6**	**5**
Fermentable reducing substances						
Liver	220	51	104	4.1	34	19
"	**119**	**56**	**83***	**4.2**	**10**	**7**
Muscle	42	4	17	1.0	36	19
"	**23**	**0**	**14**	**1.0**	**10**	**8**
Kidney	82	15	53	2.0	35	19
"	**78**	**29**	**53**	**2.4**	**10**	**8**
Blood	92	59	74	1.7	11	12
"	**86**	**69**	**76**	**1.7**	**6**	**5**
Non-fermentable reducing substances						
Liver	59	0	26	1.9	34	19
"	**57**	**18**	**34***	**2.5**	**10**	**7**
Muscle	45	4	17	1.2	36	19
"	**28**	**7**	**16**	**1.7**	**10**	**8**
Kidney	37	5	17	1.1	35	19
"	**153**	**13**	**49***	**8.8**	**10**	**8**
Blood	8	2	5	0.4	11	12
"	**11**	**4**	**7***	**0.6**	**6**	**5**

* Statistically significant by the *t* test of Fisher (10).

its administration. In most instances the feeding of the pentose resulted in significant decreases of these substances. Significant

increases occurred only in the non-fermentable reducing substances of the liver, kidney, and blood.

l-Xylose is very poorly absorbed from the gastrointestinal tract and practically all rats developed severe diarrhea after it was given. In order to overcome this diarrhea, 1 cc. of a 25 per cent *l*-xylose solution was given in place of the customary 2 cc. for the absorption studies. The coefficient of absorption is extremely low, 0.007, the average for eleven rats.

DISCUSSION

There is nothing in the data to indicate that *l*-xylose is glycogenic. A slight increase was observed in the liver glycogen of females, while there was almost a 50 per cent decrease in the livers of males. Why such a decrease occurred is problematical. There were decreases in the muscle glycogen of both males and females, also in the lactic acid content of the liver and blood, and in the fermentable reducing substances of the liver. That some of the xylose was absorbed may be adduced from the significant increases in the non-fermentable reducing substances of the liver, kidney, and blood. The rate at which it was absorbed from the gastrointestinal tract was only 7 mg. per 100 gm. of rat per hour, the lowest coefficient of absorption recorded for any carbohydrate.

The metabolism of *l*-xylose follows quite closely that of *d*-xylose with one notable exception; an unquestionable rise in the fermentable sugar of the blood occurs after the administration of the latter. The coefficient of absorption for *d*-xylose is 74, which would mean that considerably more of this pentose is absorbed than of the *l* form, as shown by the much greater rise in the non-fermentable reducing substances of the liver, muscle, kidney, and blood with the former. The significant increases in the non-fermentable reducing substances after the feeding of *l*-xylose are relatively slight when compared with the 5-fold increase for blood, 6-fold for liver, and 14-fold for kidney after *d*-xylose administration. Such differences may be explained by assuming that the gastrointestinal mucosa is not permeable to the *d* and *l* forms of the pentose to the same degree. Similar results have been noted for *d*- and *l*-xylulose, the former having a coefficient of absorption of 131 and the latter of 44.

These findings are difficult to reconcile with Verzár's theory (11) of selective absorption according to which glucose and galactose are selectively absorbed because they are phosphorylated in the intestine, while the pentoses, which are not susceptible of phosphorylation, are absorbed by simple diffusion. The absorption of the latter is, therefore, dependent on concentration. Verzár and his associates found that xylose, arabinose, and rhamnose were absorbed from the intestine at practically an identical rate which was not influenced by adrenalectomy or poisoning with monoiodoacetic acid or phlorhizin, which inhibit phosphorylation of the hexoses. According to Deuel and his collaborators (12), the rate of glucose absorption was not decreased after adrenalectomy provided the normal physiological salt balance was maintained and they concluded that the adrenal cortex did not directly alter the ability of the rat to absorb glucose. Klinghoffer (13) found decreased absorption of xylose as well as glucose after monoiodoacetic acid poisoning and attributed it to the severe intestinal pathology caused by the poisoning rather than to any specific action of the drug on intestinal phosphorylation. Lambrechts (14) concluded that the effect of phlorhizin was due to its toxic action on cellular structure instead of any specific inhibition of phosphorylation.

If we accept Verzár's view of diffusion of the pentoses, we should obtain identical coefficients of absorption[3] for *d*- and *l*-xylose and *d*- and *l*-xylulose instead of those found; namely, 0.074, 0.007, 0.131, and 0.044. It would be difficult to ascribe these differences to phosphorylation. It would seem that in any absorption study not only phosphorylation and diffusion must be considered but also the configuration of the substance in question. The results which we have obtained with optical isomers of aldo- and ketopentoses indicate that diffusion is dependent upon the structure of the sugar.

SUMMARY

The feeding of *l*-xylose to rats increased significantly the non-fermentable reducing substances of the liver, kidney, and blood,

[3] These absorption studies were all carried out at the same concentration, 2 cc. of a 25 per cent solution, with the exception of *l*-xylose, in which instance the dose was reduced to 1 cc. to prevent the diarrhea caused by the larger amount.

but failed to increase the glycogen content of the tissues. Significant decreases occurred in liver and blood lactic acid, muscle glycogen, and in the fermentable reducing substances of the liver after its administration.

l-Xylose is very poorly absorbed from the gastrointestinal tract. Its coefficient of absorption, 0.007, is the lowest recorded for any carbohydrate.

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STUDIES ON PLASMA PHOSPHATASE ACTIVITY IN RELATION TO FAT METABOLISM IN RATS

BY LEOPOLD WEIL AND MARY A. RUSSELL

(*From The Biochemical Research Foundation of the Franklin Institute, Philadelphia*)

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Extensive literature exists on the significance of plasma phosphatase in various biological problems. These reported investigations have proved to be very valuable clinically in the diagnosis of certain diseases which are connected with an increase of the plasma phosphatase activity. Although the usefulness of the tests cannot be denied, no satisfactory explanation has been given for the phenomena on which the tests are based. It seems important therefore to investigate the function of this enzyme not only in pathological but also in normal cases.

The work of Bodansky (1) on serum phosphatase of dogs has indicated the functional importance of plasma phosphatase. He found that prolonged fasting greatly reduced the serum phosphatase activity of young dogs, and an increase of this enzyme was obtained only after ingestion of carbohydrates. Ingestion of fat (cream) or meat did not have any effect. The suggestion was made that the increase of serum phosphatase after ingestion of carbohydrates was due to increased functional activity, with transitory hexose phosphate formation and production of phosphatase by the intestinal mucosa and probably by other organs, a conception which appears to be supported by the works of Lundsgaard (2) and Verzár and coworkers (3, 4) on carbohydrate resorption. However, our findings on albino rats present an entirely different picture, whereby we came to the conclusion that certain unsaturated fatty acids are the factors influencing plasma phosphatase activity.

EXPERIMENTAL

Albino rats were used. Besides other advantages (5), the plasma phosphatase activity is unusually high in rats (6), which makes them especially suitable for this type of work. Because of the limited blood supply of these animals, however, it was necessary to use an ultra micromethod which enabled the carrying out of enzymatic determinations with 1 drop of blood. The Linderstrøm-Lang and Holter technique (7) was used in combination with the capillary technique developed by Richards and coworkers (8, 9). The average deviation observed by Walker (9) was ±2.5 per cent. When our combined technique was applied to known amounts of phosphate, the average deviation was found to be ±3.0 per cent, which may be considered satisfactory for the purpose.

The general procedure was as follows: The tail of the rat was dipped into water at about 50°. A small section of the tail was cut off and a few drops of blood were squeezed out and drawn up in a thick walled capillary tube 20 mm. long with an inside diameter of 1.5 mm. One end of the capillary tube was drawn out to a narrow tip and at the point a calibration mark of 15 c.mm. was made. Before these tubes were used, they were charged with the necessary amount of potassium oxalate to prevent coagulation of the 15 c.mm. of blood. The walls of the capillary tube were coated with the dry oxalate by drawing up the measured amount of K oxalate solution and carefully drying the tube.

After the blood samples were taken, the ends of the tubes were closed with a rubber band and placed in a small Lundgren centrifuge (3000 R.P.M.) to sediment the blood cells. 3 c.mm. of the supernatant plasma then were pipetted off with a capillary pipette (7) and measured into a micro test-tube (25 mm. long and 3 mm. inside diameter) which was previously charged with 21 c.mm. of distilled water. This dilution (1:7) was necessary because of the high enzyme concentration. 7 c.mm. of the diluted plasma were taken for one determination, corresponding to 0.87 c.mm. of undiluted plasma, and pipetted into a micro test-tube. To that either 7 c.mm. of veronal-HCl buffer at pH 9.0 (10) or 7 c.mm. of the same buffer containing 0.015 M $MgCl_2$ were added; this concentration of Mg ions was found to be sufficient to produce the maximum activation. After the addition of 7 c.mm. of 0.1 mole

of sodium β-glycerophosphate the final Mg ion concentration was 0.005 M.

The mixing was carried out by the use of magnetic stirring (7). In the blank determination the substrate was placed as a drop on the wall of the micro test-tube and was incubated for the same length of time as the main determinations and mixed immediately before the reaction was stopped. Only one blank determination was made for the initial and full activity (with Mg), since it was found that the blanks were the same whether Mg was present or not. The open micro test-tubes prepared this way were placed in a copper micro test-tube holder and were incubated for 4 hours at 37° in chloroform and water vapor as described in our previous paper (5). After this incubation time the micro test-tubes were placed in ice water to stop the reaction. Then 10 c.mm. of 10 per cent trichloroacetic acid were added and the precipitate centrifuged. 15 c.mm. of the supernatant fluid (corresponding to 0.42 c.mm. of plasma) were pipetted off and transferred to a clean micro test-tube. The inorganic phosphate liberated during the incubation period was estimated colorimetrically according to the method of King (11). Into 15 c.mm. of protein-free fluid, 7 c.mm. of 5 per cent ammonium molybdate containing 15 per cent by volume of H_2SO_4 were pipetted. The aminonaphtholsulfonic acid solution prepared as described by King (11) was diluted 1:3 and 5 c.mm. of this were placed as a drop on the wall of the micro test-tube. The standard tests were prepared the same way.

Standard phosphate solutions with increasing phosphorus concentrations were made in such a way that the phosphorus concentration of 15 c.mm. of standard differed by 0.04 γ of P; standards with low phosphorus content differed from each other by only 0.02 γ of P. In order to have the same acidity as in the main determination the standard solutions contained 3.3 per cent of trichloroacetic acid.

Micro test-tubes containing 15 c.mm. of standard solutions covering a concentration from 0.02 to 1.00 γ of P were treated the same way as the main determinations. After the preparations were completed, both in the main and standard series, the 5 c.mm. of aminonaphtholsulfonic acid which was placed as a drop on the wall of the micro test-tube to prevent premature mixing were

shaken with the reaction mixture by a gentle swinging movement and the mixing was completed by magnetic stirring. In this way the color development started at the same time in all micro test-tubes; this factor is essential, as was emphasized by Walker (9). To compare the colors of the standards with the colors of the unknown solutions, the colored solutions were drawn up in standard capillary tubes with an inside diameter of 0.65 mm., an outside diameter of 0.8 mm., and a length of about 30 mm. The capillary tubes used were mechanically drawn and calibrated for constancy of diameter. The method used for this purpose was as follows: A known amount of mercury was drawn into the capillary tube about 60 mm. long. By moving the mercury column at the various portions of the capillary tube its length was measured. Any variation in the length of the mercury column should indicate a variation in the diameter of the capillary tube. From the length and weight of the mercury column the diameter could readily be calculated.

The maximum variation allowed was 2 per cent. To make the measurement of changes in the length of the mercury column more exact, the magnified image of the column was projected on graph paper and in this way the smallest changes in the length of the column due to irregularities in the diameter could be measured easily.

After the colored solutions containing the unknown and the standard phosphate were drawn up in the capillaries, the ends of the tubes were sealed with Duco cement. The standard tubes were then placed on the translucent plate of a safelight such as is generally used in photography. For illumination a 15 watt lamp was used. A labeled ebonite plate with suitable holes was used to keep the standard tubes in order. The comparison of the colors of the unknown with the standard was carried out as described by Walker (9). The shades of colors always could be distinguished easily. Only a fraction of the reaction mixture (15 c.mm. of the phosphate-containing mixture + 7 c.mm. of molybdate + 5 c.mm. of reducing agent) was used for the actual color composition, although the values given in Tables I to V present the values for 27 c.mm. We also found it very helpful for the comparison of colors, as did Richards and coworkers (8), to cover the capillary tubes with a piece of white paper in which

a rectangular window had been cut, so that the visible columns were of the same length.

Rats weighing about 200 gm. each were used. During fasting periods water was available at all times. Each substance tested was administered by stomach tube, unless otherwise specified, after 24 hours of fasting. 2 cc. of the compound in liquid form

TABLE I

Influence of Fasting on Plasma Phosphatase Activity, Expressed in γ of Phosphorus

		Activator		Activator	
		None	Mg'	None	Mg'
		Rat I		Rat II	
	hrs.				
Fasting	0	0.24	0.54	0.34	0.84
	4	0.24	0.52	0.32	0.80
	8	0.20	0.40	0.26	0.62
	16	0.12	0.28	0.14	0.36
	24	0.12	0.26	0.16	0.32
Feeding (Purina Dog Chow)	1.5	0.12	0.24	0.18	0.32
	4	0.16	0.30	0.24	0.46
	8	0.20	0.42	0.28	0.66
	16	0.24	0.48	0.32	0.72
	24	0.26	0.56	0.36	0.80
		Rat III		Rat IV	
Prolonged fasting	0	0.24	0.66	0.28	0.58
	16	0.08	0.20	0.14	0.36
	24	0.08	0.18	0.10	0.30
	40	0.08	0.16	0.10	0.24
	48	0.08	0.14	0.10	0.22
	64	0.10	0.18	0.10	0.24
	72	0.08	0.18	0.12	0.26
	84	0.10	0.24	0.14	0.28
	120	0.10	0.24	0.12	0.30

were given and 1.5 gm. in solid form made up to 3 cc. in solution or suspension, depending on the solubility of the material in water. Plasma phosphatase activity was measured at intervals during fasting and also after feeding was resumed.

Influence of Fasting on Plasma Phosphatase Activity—As fasting continued, the plasma enzyme activity fell markedly, reaching the

lowest level in about 16 hours (see Table I). After feeding was started, the enzyme activity was gradually restored, reaching the original starting value in about the same length of time that had been necessary to reach the lowest point. The changes occurring in the plasma phosphatase activity with Mg″ ions as activator parallel those occurring without Mg″ ions. Since this parallelism was found in all subsequent experiments, it was deemed unnecessary to list the initial activity (without Mg″) in Tables II to V.

The low phosphatase values obtained after 1 to 5 days of fasting did not change significantly when the fasting was extended, as is also indicated in Table I. Whether this residual enzyme activity was of different origin from the one influenced by fasting has not yet been determined. The loss in the body weights during 5 days of fasting was about 24 per cent.

Influence of Ingestion of Various Substances on Plasma Phosphatase Activity—The plasma phosphatase activity of the rats is closely linked with the resorption or utilization of the food (Table I). In order to determine which part of the diet is responsible for the marked change in the plasma enzyme activity the Purina Dog Chow was pulverized and completely extracted with alcohol-ether (1:1) and the solvent was eliminated from both fractions *in vacuo*. The soluble fraction contained chiefly the fats, cholesterol, and phospholipids, while the insoluble fraction contained mainly the carbohydrates and proteins. Only the alcohol-ether-soluble fraction had the ability to restore the low plasma phosphatase activity produced by 24 hours fasting, while the insoluble fraction was completely inactive in this respect, leaving the plasma phosphatase activity at the fasting level (see Table II). Whether this low enzyme value produced by fasting could also be achieved without fasting, simply by eliminating from the diet the alcohol-ether-soluble fraction which has the ability to restore it, was easily answered by changing the feeding of the rats from the original to the extracted food.

The high plasma phosphatase values during the feeding of unextracted food were rapidly lowered by the feeding of extracted food (see Table II). The lowest value, which was reached in about 16 hours, resembles very much the effect reached by fasting. The low plasma phosphatase values of rats can be obtained there-

fore not only by fasting but by the simple elimination from the food of the fraction which is responsible for the increase in the phosphatase activity.

The results of these experiments indicate that the fraction responsible for the increased plasma phosphatase activity is of lipid nature. In order to ascertain the activity of other constituents of the food, carbohydrates and proteins, the following substances were tested: starch, glycogen, α-glucose, maltose,

TABLE II

Influence of Ingestion of Alcohol-Ether-Soluble and Insoluble Fractions of Rat Food on Plasma Phosphatase Activity, Expressed in γ of Phosphorus

		Purina Dog Chow		Alcohol-ether-insoluble fraction of Purina Dog Chow		Extract from Purina Dog Chow	
		Rat I	Rat II	Rat III	Rat IV	Rat V	Rat VI
	hrs.						
Fasting	0	0.72	0.92	0.96	0.70	0.84	0.84
	24	0.28	0.32	0.48	0.34	0.48	0.34
Feeding	1.5	0.32	0.44	0.44	0.24	0.60	0.48
	4	0.52	0.68	0.44	0.30	0.68	0.56
	8	0.60	0.84	0.48	0.32	0.70	0.80
	24	0.76	0.98	0.44	0.28	0.96	0.90
		Rat VII	Rat VIII	Rat VII	Rat VIII		
Feeding (no fasting period)	8	0.70	0.90				
	24	0.70	0.84				
	1.5			0.68	0.72		
	4			0.48	0.60		
	8			0.40	0.52		
	24			0.30	0.40		
	32			0.28	0.36		

galactose, xylose, casein, egg albumin, edestin, and gluten. None of these had the ability to restore the plasma enzyme activity after ingestion.

Bodansky (1) reported an increased serum phosphatase activity after ingestion of carbohydrates by young dogs. In rats 5 weeks old, fed with sucrose, we failed to observe any positive reaction.

Since the ingestion of carbohydrates and proteins was shown to be entirely ineffective in restoring the plasma phosphatase ac-

tivity, experiments were conducted to determine which part of the lipid fraction is responsible for this phenomenon.

Glycerol, an essential part of fats and phospholipids, was found to be without any action. The same was true of β-glycerophosphate (neutralized) and also of cholesterol, indicating that the fatty acid part of the lipid molecule may be the decisive factor. The following saturated fatty acids and some of their derivatives were tested: propionic, butyric, tributyrin, β-hydroxybutyric, valerianic, caproic, caprylic, pelargonic, capric, lauric, myristic, palmitic, methyl palmitate, ethyl palmitate, stearic, *n*-propyl stearate, and cerotic acids. Palmitic, stearic, and cerotic acids were also tested in combination with 0.3 gm. of sodium taurocholate as emulsifying agent. The acids from propionic to lauric were administered in neutralized form because of their acidity. None of the investigated saturated fatty acids had any influence on the plasma phosphatase activity.

The investigation of Verkade *et al.* (12–14) and Flaschenträger *et al.* (15, 16) on the ω oxidation of fatty acids called attention to the importance of the dicarboxylic acids. The ingestion of malonic, succinic, glutaric, adipic, suberic, and sebacic acids (in neutralized form), however, proved to be ineffective in restoring the plasma enzyme activity.

The importance of the unsaturated fatty acids in the general metabolism of the rat was emphasized by various workers (17–22). Among the unsaturated fatty acids tested, but not listed in Table III, crotonic, fumaric, itaconic, undecylenic (in neutralized form), and chaulmoogric acids were found to be inactive. Undecylenic acid (neutralized) alone was found to be toxic to the rats and therefore a fat-free diet (see Table IV) containing 7 per cent of the neutralized acid was fed to the rats and found to be non-toxic.

The results obtained with various other unsaturated fatty acids and their derivatives are presented in Table III. Elaidic acid, ricinoleic acid, and oleicamide were found to be only slightly active in restoring the plasma phosphatase activity. Elaidic acid which was prepared from oleic acid was recrystallized and the melting point of this compound indicated that the slight activity observed after ingestion was not due to traces of oleic acid. Oleicamide was prepared according to the method of Bruson (23). Hexabromolinolenic acid and oleyl alcohol were found to have

no effect. The latter was prepared by reduction of butyl oleate (24). Only oleic, erucic, linoleic, and linolenic acids[1] proved to be strongly active in restoring the plasma phosphatase activity, a rather limited number but the number will probably increase

TABLE III

Influence of Ingestion of Unsaturated Fatty Acids and Their Derivatives on Plasma Phosphatase Activity, Expressed in γ of Phosphorus

	Fasting period		Feeding period			
	0 hr.	24 hrs.	2 hrs.	4 hrs.	8 hrs.	24 hrs.
Oleic acid	0.90	0.46	0.50	0.54	0.60	0.84
" " + sodium taurocholate	0.96	0.46	0.48	0.50	0.60	0.88
Oleicamide + sodium taurocholate	0.64	0.30	0.32	0.42	0.42	0.42
Oleyl alcohol	0.78	0.22	0.16	0.14	0.10	0.06
Elaidic acid	0.54	0.26	0.26	0.24	0.24	0.34
" " + sodium taurocholate	0.76	0.28	0.26	0.28	0.30	0.40
Ricinoleic acid	1.20	0.34	0.36	0.42	0.42	0.48
Linoleic acid	0.96	0.44	0.36	0.34	0.36	0.80
Linolenic "	0.78	0.30	0.32	0.36	0.42	0.70
" + sodium taurocholate	0.80	0.28	0.28	0.30	0.48	0.70
Hexabromolinolenic acid + sodium taurocholate	1.06	0.40	0.40	0.30	0.28	0.36
Erucic acid	0.72	0.28	0.34	0.36	0.46	0.98
Olive oil	0.80	0.36	0.38	0.42	0.56	0.90
Lard	0.70	0.18	0.16	0.36	0.40	0.68
Linseed oil	0.80	0.42	0.40	0.34	0.30	0.74
Cod liver oil	0.72	0.28	0.38	0.54	0.56	0.68
Chaulmoogra oil	0.70	0.24	0.24	0.32	0.34	0.40
Lecithin	0.80	0.34	0.30	0.24	0.24	0.16
"	0.86	0.36	0.32	0.26	0.24	0.18
Cephalin	1.20	0.54	0.30	0.30	0.54	1.08
"	0.98	0.32	0.30	0.32	0.50	0.78

when we are able to test more of these acids. Lecithin and cephalin were prepared from calf brain according to a slight modification of the method of Levene and Rolf (25) by which the yield was increased considerably.[2] Elementary analysis and melting

[1] Oleic, linoleic, and linolenic acids were identified by means of iodine number determination.

[2] The details of this method will be published later.

point determinations were made for the characterizations of these phospholipids. Repeated experiments have shown that lecithin was ineffective but cephalin was very active (see Table III).

With the exception of chaulmoogra oil, all of the oils tested were active in restoring the plasma phosphatase activity, as may be seen from Table III. The only slight activity of chaulmoogra oil is not surprising in view of the negative results obtained with chaulmoogric acid. No activation of the plasma phosphatase

TABLE IV

Influence of Ingestion of Saturated and Unsaturated Triglycerides Added to Fat-Free Diet on Plasma Phosphatase Activity, Expressed in γ of Phosphorus

Diet	Feeding period	Supplementary diet* No.			
		I	II	III	IV
	days				
Purina Dog Chow	2	1.16	0.78	1.10	0.66
	4	0.98	0.86	1.00	0.60
	6	1.10	0.84	1.10	0.70
Fat-free diet + 7 per cent saturated triglycerides	2	0.48	0.20	0.24	0.30
	4	0.48	0.20	0.44	0.22
	6	0.52	0.18	0.36	0.20
Fat-free diet + 7 per cent olive oil	3	0.84	0.54	0.78	0.60
	4	0.84	0.74	0.82	0.58
	6	1.10	0.72	0.86	

* The 7 per cent saturated triglyceride of Diet I was trimyristin; of Diet II tripalmitin; of Diet III tristearin; of Diet IV tristearin + 7 per cent cholesterol.

activity was observed when unsaturated oil emulsions were added directly to the plasma.

Since in our experiments pure fats or fatty acids had been fed and ketonuria had resulted, it was desirable to determine whether a carbohydrate-abundant diet, which eliminates ketonuria, would influence the plasma phosphatase activity. A fat-free diet was made up of 240 parts of casein, 721 parts of sucrose, 39 parts of McCollum-Davis salt mixture (26), and 6.5 parts of yeast extract. The casein and yeast preparation was extracted for 24 hours with alcohol-ether 1:1 before use. To the fat-free diet 7 per cent of trimyristin, 7 per cent of tripalmitin, or 7 per cent of tristearin

was added. The rats were fed 6 days on Purina Dog Chow, afterward 6 days on the fat-free diet plus 7 per cent of saturated triglycerides, and finally on the fat-free diet plus 7 per cent of olive oil as unsaturated triglycerides. The possible influence of

TABLE V

Relation of Amount of Fat Present in Diet Ingested to Plasma Phosphatase Activity, Expressed in γ of Phosphorus

	No. of feeding days	Rat I	Rat II	Rat III	Rat IV
Purina Dog Chow	2	0.70	0.66	0.78	0.80
	3	0 76	0.78	0.60	0.74
	4	0.68	0.70	0.70	0 74
Diet without lard	1	0.34	0 26	0.24	0.40
	3	0.24	0.14	0.16	0.18
	4	0.22	0.10	0 14	0.18
" + 0.5% "	2	0.26	0.12	0.20	0.20
	3	0.22	0.12	0.16	0.22
	4	0.28	0.14	0.24	0.22
" + 1.0% "	2	0.34	0.18	0.24	0 36
	3	0.36	0.32	0.32	0.28
	4	0.38	0.34	0.32	0 30
" + 2% "	1	0 46	0.36	0.34	0.32
	3	0.40	0.46	0.42	0.46
	4	0.48	0.50	0.44	0.42
" + 3% "	1	0.54	0.48	0.52	0.50
	2	0 56	0.50	0.48	0.46
	4	0 48	0.48	0.48	0.46
" + 5% "	2	0.52	0.56	0.60	0.58
	3	0.56	0.66	0.62	0.64
	4	0.60	0 66	0.70	0.62
" + 8% "	1	0.68	0.78	0.70	0.78
	3	0.78	0.72	0.72	0.74
" + 12% "	2	0.76	0.64	0.66	0.70
	3	0.68	0.68	0.72	0.74
	4	0.68	0.72	0.68	0.72
100% lard	1	0.74	0.70	0.72	0.68

cholesterol on the saturated triglycerides was also studied by mixing the fat-free diet with 7 per cent of tristearin and 7 per cent of cholesterol; the results are presented in Table IV. The ingestion of the diet containing the saturated triglycerides greatly

lowered the plasma phosphatase activity which was quickly restored when olive oil was added to the fat-free diet, an effect similar to that obtained when saturated or unsaturated fatty acids were fed alone. The presence of cholesterol in combination with tristearin in the fat-free diet did not influence the enzyme activity (Table IV).

To investigate the relation between the amount of fat in the diet and the phosphatase activity of the plasma the fat-free diet, with increasing amounts of lard, was fed. To obtain uniform distribution the lard was dissolved in ether, mixed with the diet, and dried. Lard was used to prevent any deficiency disease produced by the absence of certain unsaturated fatty acids (17, 20, 21) which are present in the lard. The results obtained are given in Table V. The plasma phosphatase activity dropped markedly when the diet was changed from Purina Dog Chow to the fat-free diet. An increase in lard content of the diet produced a gradual increase in the plasma phosphatase activity. The original high enzyme level was reached when the lard content of the diet was about 8 per cent. If we take into consideration that only about 65 per cent of the fatty acids present in the lard is unsaturated, the 8 per cent of lard is equal to 5 per cent of unsaturated fat. A further increase in the lard concentration did not increase the plasma phosphatase activity even if 100 per cent of lard was fed.

DISCUSSION

The presence of phosphatase in the plasma of albino rats was shown by these investigations to be due to the ingestion of a certain part of the lipid fraction of the food. The ingestion of carbohydrates or of proteins did not result in an increased plasma phosphatase activity. An increase in the activity of the plasma enzyme occurred only when unsaturated fats or fatty acids were added to the diet. The saturated fatty acids and also the saturated dicarboxylic acids proved to be entirely ineffective. The experiments carried out with palmitic, stearic, and cerotic acids in combination with sodium taurocholate, however, indicated that when the resorption was increased by emulsification the enzymatic picture was not changed and the observed low phosphatase values were not due to the inability of the intestinal mucosa to resorb these acids.

The ineffectiveness of chaulmoogric acid and the slight activity of ricinoleic acid are possibly due to their diarrheal action, and also possibly to the position of the double bond of the former acid. When the action of linolenic acid is compared with hexabromolinolenic acid, the importance of the double bond becomes apparent. The degree of unsaturation seems to be of no decisive importance, since oleic, linoleic, and linolenic acids were all active. From the results shown in Table III it becomes apparent that in order to mobilize plasma phosphatase a certain molecular magnitude of the unsaturated acids is required. The unsaturated acids with the carbon chain of C_3, C_4, C_5, and C_{11} were inactive. The next available acid, oleic acid with the carbon chain of C_{18}, was active. To determine the exact length of the carbon chain of the unsaturated fatty acid at which point activity begins requires further investigations. It is of interest to compare the action of oleic acid with elaidic acid; the latter one is a geometric isomer of the former one. The higher melting point of 44–45° is not sufficient to explain its slight activity compared with the marked activity of oleic acid, since emulsification with sodium taurocholate did not improve this ratio. It seems possible that a certain natural configuration is necessary to produce this reaction. The negative results obtained after ingestion of oleyl alcohol and the only slight effectiveness of oleicamide clearly demonstrated the necessity of the free carboxyl group. The slight activity of oleicamide may be due to a slow decomposition of this compound in the body. The high effectiveness of cephalin and the inactivity of lecithin are rather surprising, since both contain unsaturated fatty acids. Factors responsible for this phenomenon, however, require further investigation.

The mechanism of fat resorption has been extensively studied (27–30) and strong evidence presented that the absorbed fatty acids are transformed into phospholipids within the intestinal mucosa as an essential step in the resynthesis of neutral fats. The phospholipids formed in this way are transported by the plasma (31–33) to the various organs where they may be deposited or transformed to neutral fats to be decomposed according to the need of the body. It is probable that the increased plasma phosphatase activity following the ingestion of unsaturated fatty acids may be linked with the process of transformation of phos-

pholipids to neutral fats. The fact, however, that the ingestion of only certain unsaturated fatty acids is connected with an increased plasma phosphatase activity suggests the possibility that a difference may exist between the metabolism of saturated and of unsaturated fatty acids.

SUMMARY

A colorimetric micromethod was developed for the estimation of plasma phosphatase activity in less than 1 c.mm. of plasma. The plasma phosphatase activity of albino rats was found to be greatly reduced by fasting. Only the ingestion of the alcohol-ether-soluble fraction of the rat diet increased the plasma phosphatase level. The ingestion of carbohydrates and proteins did not increase the low plasma phosphatase activity produced by fasting. Ingestion of saturated fatty acids and saturated dicarboxylic acids gave the same results as ingestion of carbohydrates or proteins. An increase in the low plasma phosphatase activity produced by fasting resulted only from the ingestion of certain types of unsaturated fatty acids, wherein a certain molecular magnitude, the double bond in the carbon chain, and the free carboxyl group are essential. The enzyme reaction following the ingestion of various fatty acids was not influenced by the presence of carbohydrates or proteins. Cephalin ingested after fasting produced a marked increase in the plasma phosphatase activity, while under the same conditions lecithin was found to be entirely without action. For obtaining the highest plasma phosphatase activity about 8 per cent of lard was required in the diet. A further increase in the lard concentration did not increase the enzyme activity.

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A MODIFIED SALICYLALDEHYDE METHOD FOR THE DETERMINATION OF ACETONE BODIES IN BLOOD AND URINE

By JEANETTE ALLEN BEHRE

(*From the Department of Chemistry, Northwestern University Medical School, Chicago, and the Research Laboratory, Union Central Life Insurance Company, Cincinnati*)

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Colorimetric methods for the determination of acetone based on the reaction of acetone with salicylaldehyde in alkaline solution hold an intermediate position, with respect to specificity and sensitivity, between gravimetric and iodometric methods (1). In these respects they appear to have no advantage over recently published methods (2, 3) which combine the specificity of mercury precipitation with a high degree of sensitivity. However, because of their greater rapidity and simplicity of technique salicylaldehyde methods may frequently be preferred.

In previous salicylaldehyde methods use has been made of a boiling water bath (4, 5), or of a bath at 45–50° (6, 7), to hasten color development. The former procedure has been criticized (7) because of the loss of small amounts of acetone at this high temperature, while the latter procedure is slower and less convenient. In the present paper a modification is proposed which reduces the time necessary for the development of color without the application of heat. The speed with which the salicylic aldehyde reaction takes place depends upon the concentration of reacting substances as well as upon temperature. If the concentration of reagents exceeds a certain point, a precipitate is formed which has hitherto been a limiting factor. In the present method the concentration of the reacting mixture is increased and the precipitate which forms is dissolved after the reaction is complete.

Preliminary Procedures—The colorimetric determination of acetone, preformed or from the other acetone bodies, is made on a distillate from blood filtrate or urine. Except in the determination of urinary acetone and diacetic acid the sensitivity of the method

is limited by the procedures involved in the preparation of these distillates. These procedures, as slightly modified from those previously used (4, 5), are briefly as follows:

An all-glass distilling apparatus is recommended, especially for the determination of β-hydroxybutyric acid.[1] This is not essential and well fitting corks, but no rubber, may be used for connections. The apparatus consists of a 200 to 300 cc. distilling flask with dropping funnel, a 200 mm. water-cooled condenser and delivery tube, and a receiving tube or flask for which it is convenient to use a 15 cc. graduated centrifuge tube for acetone and diacetic acid and a 200 cc. round bottomed flask for β-hydroxybutyric acid. The enlarged part of the delivery tube should rest in the top of the receiver, forming a cover for it, and the lower end is drawn out to a fine tip which reaches to the bottom of the receiver, where it is covered by a minimum amount of water. Glass beads in the distilling flask and a microburner, with no sand bath, are used. Glass joints are lubricated with water. Heat is applied slowly at the beginning of distillation to prevent violent bubbling in the receiving tube.

For the determination of acetone and diacetic acid a volume of blood filtrate or urine (15 to 30 cc.) is acidified with sulfuric acid and distilled to a volume of distillate equal to one-third, or more, of the original volume.

For the determination of β-hydroxybutyric acid in urine interfering substances are removed by the Van Slyke copper sulfate-calcium hydroxide procedure (8). All volumes may be reduced proportionally if advisable. If the urine is dilute, its volume may be increased in relation to the volume of the final mixture, twice the amount of copper sulfate (in 40 per cent solution) and calcium hydroxide being used. After standing for 30 to 45 minutes the mixture is filtered, or centrifuged and filtered. An aliquot of the filtrate is transferred to the distilling flask and acidified with sulfuric acid. Water is added, if necessary, to make the volume not less than 30 cc. The dropping funnel is inserted and one-third, or more, of the volume is distilled to remove acetone and diacetic acid, leaving a volume of 20 to 60 cc. (approximately) in the distilling flask. Since some acetone is lost during the copper-calcium treatment, a separate distillation should be made

[1] An apparatus of this sort, with interchangeable ground glass joints, has been made for the writer by Eck and Krebs, New York.

from untreated urine to determine acetone and diacetic acid. It has been found possible to reduce the time for oxidation and distillation of β-hydroxybutyric acid to half that prescribed by the Hubbard method[2] (9). This alteration reduces the volume of distillate and consequently increases the sensitivity of the method. The solution is brought to a boil and 15 cc. of sulfuric acid (concentrated, diluted 1:1) and 10 cc. of 0.2 per cent potassium dichromate are added by drops during the first 5 minutes of distillation, followed by 25 cc. of the dichromate during each of the following 5 minute periods. The rate of distillation is regulated so that from about 50 to 85 cc. are distilled at the end of 15 minutes.

β-Hydroxybutyric acid in blood filtrate is determined in the same way except that the copper-calcium treatment and separate distillation of acetone and diacetic acid are unnecessary.

Colorimetric Determination

Reagents—Salicylaldehyde. Eimer and Amend, acid salicylous, synthetic; C.P.

Potassium hydroxide, C.P. A saturated, aqueous solution, specific gravity 1.540.

Standard acetone solutions. As previously described (4). For accuracy the stock solution should be standardized by iodine titration. For less accurate work it can be assumed that 5 cc. of freshly opened or well preserved C.P. acetone diluted to 500 cc. give a 0.78 to 0.79 per cent solution. Artificial standards are described in Table I.

Ethyl alcohol. (For Procedure B only.) Aqueous 70 to 75 per cent solution.

Procedure—Exactly 0.1 cc. of salicylaldehyde is measured into a tube graduated to 5 and 10 cc.[3] with a 0.1 cc. pipette[4] or a

[2] The dichromate-sulfuric acid procedure, as applied in our methods, is not entirely satisfactory for the determination of small amounts of β-hydroxybutyric acid. More sensitive methods for this oxidation are at present being investigated.

[3] Tubes made by the Klett Manufacturing Company, Inc., New York, for use in the Klett-Summerson photoelectric colorimeter, graduated to 5 and 10 cc., are particularly convenient. Tubes graduated to 10 cc., of uniform bore, made by the Fales Chemical Company, Cornwall Landing, New York, for use in the Kingsbury-Clark albumin determination, are also convenient.

[4] A short, 0.1 cc. pipette made by Eimer and Amend for the Folin micro

1 cc. pipette graduated to tenths (slightly less accurate), followed by 2 cc. of the distillate or acetone standard and 1.5 cc. of the potassium hydroxide[5] from a burette with a fine glass tip. The contents of the tube are mixed by several churning motions with a glass rod flattened at one end at right angles to the rod to form a foot of a size to fit easily into the tubes used. The rod is left in the tube which is allowed to stand at room temperature for 20 minutes or longer. After this the precipitate is dissolved by adding either (Procedure A) distilled water to 10 cc. (or more, see below), or (Procedure B) the aqueous alcohol to 5 or to 10 cc. (see below). Solution and mixture are accomplished by churning up and down briskly with the rod. Choice between Procedures A and B depends largely upon the acetone concentration, since Procedure B gives more, and Procedure A less, color. The use of both solvents widens the range of acetone concentrations which can be determined, but for certain types of work it may be satisfactory to use either one or the other exclusively. The range of concentration for which each solvent is practical is discussed in the following paragraph. During the period of color development it is possible to estimate roughly how much acetone is present and therefore which solvent is preferable. Since the two ranges overlap, the choice is significant only for very low and very high concentrations. The reading may be made in either a visual or a photoelectric colorimeter.[6] With the former, acetone standards (or artificial standards), as described below, are used and the calculation is made as usual. Micro plungers and cups are convenient for use with Procedure B. With a photoelectric colorimeter a curve for comparison may be constructed from standardized acetone solutions. For approximate results a side-to-side comparison may be made in test-tubes of uniform bore, with the artificial standards described below. After Procedure A the color

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blood sugar method has been found particularly convenient for these measurements.

[5] Potassium instead of sodium hydroxide is used in the present method because its greater solubility makes it possible to obtain a higher concentration of alkali during the reaction. Aside from this it appears to have no specific advantage over sodium hydroxide.

[6] The Klett-Summerson photoelectric colorimeter has been found satisfactory for this determination which can be carried out entirely in the standard tubes made for this instrument.

fades, and after Procedure B there is a slight and gradual increase in color. For this reason, if acetone standards are used, they should be diluted at the same time as the unknowns. If artificial standards or a photoelectric colorimeter is used readings should be made within 15 minutes after dilution by Procedure A and within 30 minutes after dilution by Procedure B.

Range of Concentration and Proportionality—Solutions which contain from about 0.5 to 20 mg. per cent of acetone[7] can be read in a visual colorimeter after addition of water to 10 cc. (Procedure A). With this instrument and procedure an exact indirect proportionality exists between acetone concentration and scale reading for all concentrations above 1 mg. per cent. Below this the readings are exact for concentrations between 0.5 and 0.75 and between 0.75 and 1.0 mg. per cent. Concentrations too dark to be read can be further diluted with water, after the reaction has taken place, so that the color falls within the reading range. The reagents used in the method are sufficient to react with 2 cc. of a 200 mg. per cent acetone solution, and dilution can be made to as much as 200 cc. without disturbing the proportionality of the reading against standards diluted to only 10 cc., provided the color determined is not less than that given by a 2 mg. per cent solution. With Procedure B concentrations of from about 0.05 to 5 mg. per cent of acetone can be read in a visual colorimeter if the volume is made to 5 cc. and a little over twice these amounts if the volume is made to 10 cc. Readings are correctly proportional to concentration if the latter is above about 0.25 mg. per cent. Below this concentration the range of proportionality is limited. Concentrations of over 0.25 mg. per cent, made to 10 cc., can be read against those made to 5 cc. With a photoelectric colorimeter the reading range depends upon the type of instrument and filter used. With a Klett-Summerson colorimeter and green filter (No. 54) the range of acetone solutions which can be read is from about 0.02 to 8 mg. per cent after water dilution to 10 cc. and from about 0.01 to 1.5 mg. per cent after alcohol dilution to 5 cc. The curve obtained by plotting acetone concentration against the logarithmic scale readings of this instrument deviates from a straight line

[7] The figures given in connection with concentration refer to the mg. of acetone in 100 cc. of the solution or distillate, 2 cc. of which are used in the determination. The amount of acetone present during the determination is, in each case, one-fiftieth of this figure.

after Procedure A, the increase in readings becoming smaller with increasing concentration. Practically a straight line curve is obtained after Procedure B (solution made to 5 cc.) between 0.1 and 1.5 mg. per cent concentrations. A more satisfactory filter might be found for this determination. The blue and red filters which have been tried were not found suitable.

Choice of Acetone Standards—If acetone standards are used, there is considerable latitude in the choice of those to be made with each set of determinations, because of the proportionality which exists between concentration and depth of color, and because of the possibility of additional dilution of either standard or unknown after the reaction has taken place. If both Procedures A and B are used, the following scheme is suggested. 0.1, 1.0, and 5.0 mg. per cent acetone solutions are made up from the stock solution, and five standard tubes are prepared from them. 1 cc. of the 0.1 mg. per cent solution and 1 cc. of distilled water are put into Tube 1, 2 cc. of the same solution into Tube 2, 2 cc. of the 1.0 mg. per cent solution into each of Tubes 3 and 4, and 2 cc. of the 5 mg. per cent solution into Tube 5. The tubes are treated according to the directions given above for the method. After the reaction has taken place, Tubes 1 and 2 are made to 5 cc., and Tube 3 to 10 cc., with the alcohol solution; Tubes 4 and 5 are made to 10 cc. with distilled water. The alcohol standards are equivalent to 0.05, 0.1, and 0.5, and the water standards to 1.0 and 5.0 mg. per cent solutions.[7] Unknowns of from 0.05 to 200 mg. per cent can be read against these standards. Unknowns obviously below 1.0 mg. per cent are made to 5 cc. with alcohol and those obviously above, to 10 cc. with water. For doubtful ones, either solvent is used. Each is read against the nearest standard dissolved by the same solvent. Unknowns of concentrations over 1.0 mg. per cent dissolved with alcohol and those of over 10 mg. per cent dissolved with water can be further diluted with the same solvent to come within the range of the 0.5 and of the 5.0 mg. per cent standards. By this scheme the scale readings are correctly proportional to the concentration for all unknowns above 0.25 mg. per cent diluted with alcohol, and above 0.75 mg. per cent diluted with water. Below these amounts there may be an error of from 5 to 10 per cent of the concentration.

Artificial color standards have been made for use after Procedure A. The value of such standards is limited by possible variations in the chromogenic capacity of different samples of salicylaldehyde, as previously discussed (10). However, in the past 9 years the writer has obtained over a dozen different samples of the Eimer and Amend product at different times, all of which gave the same color in this reaction, so that the use of artificial standards appears justified as long as this product is available. For much experimental, as well as routine, work, when great accuracy is not demanded, these standards are useful and time-

TABLE I

Directions for Making Artificial Standards for Use after Procedure A

Only C.P. products should be used. The amounts specified are made to 100 cc. with distilled water. All standards except the last two may be prepared from 10 per cent solutions of the dichromate and cobalt chloride.

Acetone equivalent	Potassium dichromate	Cobalt chloride
mg. per cent	*gm.*	*gm.*
0.5	0.065	0.64
1.0	0.095	1.40
2.0	0.60	1.80
3.0	1.80	1.70
4.0	3.30	1.60
5.0	5.00	1.50
7.5	13.30	0.80
10.0	13.50	0.30 + 8 cc. concentrated sulfuric acid

saving. The standards described in Table I were made to match acetone solutions, standardized by iodine titration and treated according to this method. They can be used for side-to-side comparisons, by transmitted light, in test-tubes of a uniform bore of approximately 13 mm., or for comparison in a visual colorimeter, with the standard set at 13 mm. They were made to match acetone solutions at this depth because this is the approximate diameter of both the Fales and Klett, uniform bore tubes which have been used in the side-to-side comparison. In a visual colorimeter the proportionality with these standards does not cover as wide a range as with acetone solutions. They have no value in a photoelectric colorimeter.

DISCUSSION

The Behre and Benedict (4) and the Behre (5) methods are subject to the criticism of Ravin (7) that traces of acetone are lost when a boiling water bath is used for the development of color, as evidenced by the qualitative test (11). Ravin also pointed out that the addition of salicylaldehyde by drops is subject to inaccuracy. The present method, to which these objections are not applicable, is felt to be preferable to either of the earlier ones, but it might be stated that in the Behre and Benedict method these inaccuracies can be greatly reduced by careful and uniform technique[8] and that in the Behre clinical method they are insignificant because of the purpose of the test and the type of colorimetric comparison used.

At least two reactions appear to be involved in the formation of maximum color with salicylaldehyde; one, which gives most of the color, is hastened by warming, while the other, which gives an additional fraction of color, is inhibited as the temperature is raised. This secondary color appears when solutions are allowed to cool after warming and almost entirely disappears if they are rewarmed. "Blank" color from the reagents alone increases on cooling but does not account for the total increase. The full color from both reactions is developed slowly at room temperature, or if the solutions are chilled to 10° and then allowed to stand at room temperature. The secondary color which develops on cooling was discussed by Ravin (7) in whose method a 30 minute cooling period is used to develop maximum color. Korenman's method (6) is similar in this respect. Behre and Benedict recognized this increase in color on cooling, but sacrificed maximum color to speed. In the present method the temperature in the tubes is raised to about 37–40° by the addition of the concentrated alkali, and drops almost to room temperature during the 20 minute standing period. The spontaneous warming and cooling favor the development of maximum color.

[8] If all tubes in a series are treated exactly alike in this method, and if standard and unknown are of nearly the same concentration, the error due to loss of acetone is very slight, and, if the same number of drops of salicylaldehyde is added to each of a series of tubes in succession, from a long pipette held in a semihorizontal position, uniform results can be obtained in any one series.

The specificity of the salicylaldehyde reaction for acetone has been studied by Braunstein (12) and Thomson (13). The latter author found the reaction more sensitive for acetone than for any of the other substances studied which gave a positive reaction. These included acetaldehyde, pyruvic acid, propionic acid, ethyl acetoacetate, and a number of other compounds. It is found that with the method described in this paper acetaldehyde, pyruvic acid, and lactic acid, in amounts occurring in biological fluids, do not interfere. A 25 mg. per cent solution of acetaldehyde gives approximately the same amount of color as a 0.2 mg. per cent acetone solution, while a 500 mg. per cent acetaldehyde solution corresponds to about 25 mg. per cent of acetone. The acetaldehyde color is yellower, less red, than the acetone color, which makes exact comparisons difficult. Pyruvic acid reacts to give about one-fiftieth as much color as acetone (the color is also yellower), but distillates from acetone and pyruvic acid show no effects from the latter. The same is true of lactic acid, which does not itself react. However, if 25 mg. of pyruvic acid are oxidized and distilled by the β-hydroxybutyric acid procedure, the distillate gives a color with salicylaldehyde equivalent to that of about 0.4 mg. per cent of acetone. The distillate from a large excess of lactic acid (600 mg.) treated in the same way gives a yellower color with salicylaldehyde than does a blank. The study of possible interference from other biological substances is being continued.

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SUMMARY

A modified salicylaldehyde method for the determination of acetone in distillates from urine or blood filtrate is described. The reaction takes place in a concentrated mixture of the reacting substances without application of heat and is complete in 20 minutes. The precipitate which forms is dissolved either in water or alcohol. The range and sensitivity of the method are somewhat increased over that of previous salicylaldehyde methods. Either

a visual or photoelectric colorimeter can be used. An almost exact proportionality exists between acetone concentration and scale reading in a visual colorimeter. Artificial color standards for visual colorimetry are also described. Procedures for the oxidation of β-hydroxybutyric acid and the distillation of acetone have been slightly modified. The specificity of the reaction is briefly discussed.

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STUDIES IN MINERAL METABOLISM WITH THE AID OF ARTIFICIAL RADIOACTIVE ISOTOPES

V. THE ABSORPTION, EXCRETION, AND DISTRIBUTION OF LABELED SODIUM IN RATS MAINTAINED ON NORMAL AND LOW SODIUM DIETS*

BY DAVID M. GREENBERG, W. WESLEY CAMPBELL, AND MAKIO MURAYAMA

(*From the Division of Biochemistry, University of California Medical School, Berkeley*)

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The two biologically essential alkali cations, sodium and potassium, follow very different paths of metabolism in the animal organism. Presumably, this is a consequence of the fact that, in general, potassium is an intracellular constituent and sodium an extracellular constituent of the animal organism. The two cations are of major importance in determining the partition of the body water (1).

A tracer study of the metabolism of potassium previously has been reported from this laboratory (2). The present paper describes the results of a tracer study of the absorption from the gastrointestinal tract, the rate of excretion via the urine, and the distribution in certain viscera of the body of sodium administered as sodium chloride to normal and mildly sodium-deprived rats.

EXPERIMENTAL

The radioactive sodium isotope, Na^{24}, with a half life of 14.8 hours (3), was prepared by the bombardment of sodium metal in the cyclotron. After bombardment the metal was washed off the target holder with 50 per cent alcohol in an atmosphere of CO_2. The washings were acidified with hydrochloric acid to insure com-

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plete solution and to convert the sodium to the chloride and then evaporated to dryness to drive off the excess hydrogen chloride. The sodium chloride was dissolved in a small amount of water and the neutral solution was treated with H_2S to precipitate traces of heavy metals that are always present and that are also radioactive. The solution was filtered through a Jena sintered glass filter and the filtrate aerated to remove any trace of H_2S. The salt then was diluted to 1 per cent with respect to sodium chloride and used in this concentration.

The rats used in these experiments were 12 to 14 weeks old. They had been placed on synthetic diets at 4 weeks of age, when

TABLE I

Composition of Basal Diet and Salt Mixtures

Thiamine chloride 5.0 mg., synthetic riboflavin 4.0 mg., nicotinic acid 50.0 mg.

Basal diet		Salt mixture		
		Salt	Control	Na-low
	gm.		*gm.*	*gm.*
Casein (acid-washed)	25	$Ca_3(PO_4)_2$	1.5	1.5
Fat (Crisco)	23	NaCl	0.75	
Sucrose	50	KCl	1.25	2.0
Cod liver oil	2.5	$MgSO_4 \cdot 7H_2O$	1.0	1.0
Rice bran extract	3.0	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.3	0.3
Salt mixture	4.8	Total	4.8	4.8

they weighed 50 to 60 gm. The tests were carried out on two groups of twelve rats each.

The composition of the basal diet used and of the control and low sodium salt mixtures is shown in Table I. The sodium content of the deficient diet was 60 mg. and of the control diet 350 mg. per 100 gm. of food. The sodium-low diet employed in these experiments was only on the border line of deficiency, and it was not expected to produce drastic deficiency symptoms such as were observed by Orent-Keiles and McCollum (4, 5), on a diet of 0.002 per cent of sodium. Osborne and Mendel (6) obtained normal growth in rats on a diet of 0.035 per cent sodium. While the sodium-low animals in the present experiments averaged about 10 per cent less in weight than the controls, the difference was

not statistically significant. On a diet of the same sodium content as ours, Anderson and Joseph (7) obtained animals that were inferior in growth and in general appearance to the control rats after 5 to 6 months on the diet.

At the time of the experiments, the rats weighed between 140 and 220 gm. The average weight of the control animals was 183 gm. and that of the sodium-low was 168 gm. All animals were fasted for 24 hours before the administration of the radioactive sodium chloride solution. Following administration of the Na*Cl, the rats were placed in wire-bottomed metabolism cages equipped with feces-urine separators (8) in order to collect the excreta separately.

Except where otherwise noted, 1 ml. of the 1 per cent Na*Cl solution was administered to each rat by stomach tube. At about the end of the desired time interval for a particular experiment, the rat was anesthetized with ether and sacrificed by withdrawing blood as completely as possible by cardiac puncture. The blood was collected over oxalate and centrifuged at once to obtain the plasma. The various organs were removed and transferred to stoppered test-tubes for weighing.

For time intervals up to about 2 hours, the stomach was opened and the contents washed out with a stream of distilled water. The contents of the small intestine were washed out by inserting a syringe at the upper end and forcing out the contents with a stream of water. The tissues were weighed into 10 ml. Coors ashing capsules, dried, and ashed in an electric muffle at 450–500°. The ash was dissolved in a minimum of dilute hydrochloric acid and evaporated to dryness in the capsule. Precautions were taken to distribute the chlorides formed as uniformly as possible over the bottom of the capsule.

To determine the radioactivity of the blood plasma, a measured portion was dried in a capsule and read without ashing. Because of the strong γ rays emitted by Na^{24}, ashing had no measurable effect on the activity.

All radioactivity measurements were made by means of a Lauritsen electroscope and the rate of discharge was compared with that of a standard consisting of 0.002 to 0.01 ml. of the original Na*Cl solution dried in another ashing capsule.

A reading was made on the standard at approximately hourly

intervals while observations were being made on the test samples. The electroscope discharge rates found for the standard, after the background rate was subtracted, were plotted against a linear time scale on semilogarithmic paper. This yields a straight line from which it is easy to determine the half life of the sodium and the value for the radioactivity of the standard at any instant. Dividing the electroscope discharge rate obtained for a test sample by the rate of the standard obtained graphically and multiplying by the appropriate dilution factor give the per cent of the tagged dose administered that is present in the particular tissues represented by the sample.

This method of calculation is much simpler than a common one of correcting all observations back to some zero time, and is easier than an arithmetical calculation of the rate of the standard at times between observed values. The observed half life also furnishes a check on the purity of the sodium sample and is a proof of its identity.

Results

Absorption—The rate of absorption may be judged from the curves of the percentage of labeled sodium remaining in the fluid contents and tissues of the stomach and small intestine. These are plotted in Fig. 1. The rate of removal of the sodium from the gastrointestinal tract, as was found with potassium, is subject to great individual variations, particularly in the early time periods.

The absorption of the sodium appears to be considerably faster than was found for potassium. Only about 25 per cent of the sodium was present in the contents and tissues of the stomach and small intestine after about 10 minutes as against an average of about 75 per cent for potassium (2). About 95 per cent of the labeled sodium disappeared from the gastrointestinal tract within an hour. These observations are in harmony with those of Hamilton (9), who measured the absorption of the biologically important univalent cations and anions in man with radioactive isotopes by determining the increase in the radioactivity of the hand.

Mild sodium deprivation has no significant effect on the rate of its absorption from the gastrointestinal tract.

No sodium accumulated in the liver during the period of absorption, such as was found in the case of potassium.

Excretion—The curves for urinary excretion of the labeled sodium are shown in Fig. 2. With doses between 0.5 and 20 mg. of administered Na*Cl given by stomach tube in 5 ml. of water, the rate of excretion was independent of the size of the dose (Curves 1 and 3). A much larger fraction of the administered sodium was excreted when 100 mg. of Na*Cl in 5 ml. of water were administered (Curves 2 and 4). The sodium-deprived rats excreted less of the equivalent doses than did the normals. This

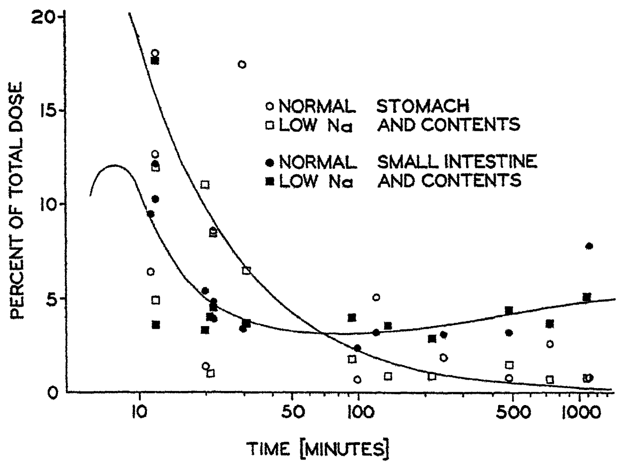

FIG. 1. Residual radioactive sodium in the gastrointestinal tract after oral administration of labeled sodium chloride (1 ml. of 1 per cent solution).

is contrary to the observation of Anderson and Joseph (7), who found an even greater excretion of sodium by the sodium-low animals. The divergent results may be due to the difference in the animals, and the experimental procedures. Anderson and Joseph's rats were maintained for 5 to 6 months on the experimental diets as against 2 to 2.5 months for our experimental animals, and a saline-glucose solution was administered to their test animals on the day prior to the experiment.

Whereas the urinary excretion of labeled potassium was found to follow a nearly linear course for a period of about 3 days after

administration, the excretion of the sodium follows an exponential curve.

Distribution—The changes in the specific content of radioactive sodium of representative body tissues with time are shown in Fig. 3. The average values and standard deviations of the specific contents of all the tissues that were studied are given in Table II.

The uptake of sodium by the tissues and body fluids is extremely rapid. In most cases the maximum accumulation was reached within the first sampling period (12 minutes). This rapidity of uptake has also been observed by Griffiths and Maegraith (10). In nearly all cases the specific accumulation remained

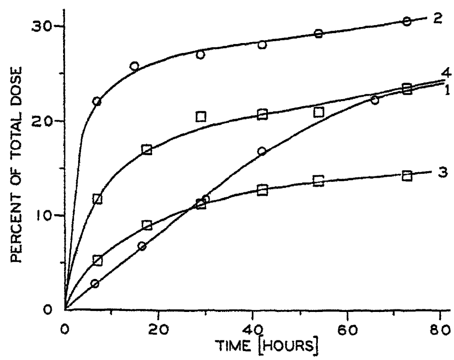

Fig. 2. Urinary excretion of radioactive sodium. Curves 1 and 3 represent doses of between 0.5 and 20 mg. of Na*Cl in 5 ml. of water, and Curves 2 and 4 represent doses of 100 mg. of Na*Cl in 5 ml. of water. The circles represent rats on control diet; the squares, rats on the sodium-low diet.

at a nearly constant level for the total period of observation (1100 minutes). The significance of the rapid distribution of the labeled sodium to the phase make-up of the tissues of the body will be brought out in the next section.

The highest concentration of labeled sodium, as would be expected, was found in the blood plasma. Fig. 3 shows that the Na* concentration is in general somewhat higher in the plasma of the animals on the low sodium diet. No such difference between the two groups was found for the calculated amounts of radioactive sodium in the whole of the blood plasma. In the calculations the value of the blood volume for the rat as given by Donaldson (11) and the directly measured hematocrit values

were used. This calculation showed an average content of 11 per cent of the administered labeled sodium to be present in the blood stream.

The skin, muscle, and blood plasma are the most important storage sites for the sodium. The former two tissues accounted for about 20 per cent each of the administered radioactive sodium. The total content of the liver was about 2.5 per cent. In the kidney, aside from several unusually high values of specific content, there is about the same constancy as was found in the liver.

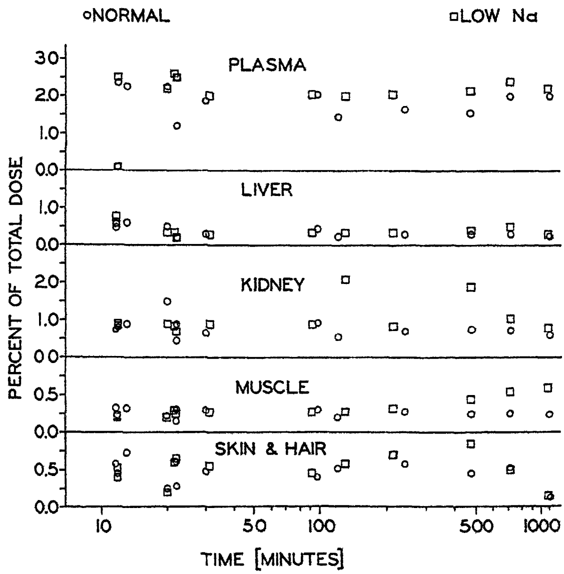

FIG. 3. Specific content (retention) of radioactive sodium in blood and in various tissues per gm. of fresh weight.

The actual values are about twice as great as those of the liver. The high values in the kidneys, presumably, are due to concentration of the labeled sodium in the process of being excreted. It is a point of interest that the unusually high values of specific sodium content in the kidneys were found in the sodium-low animals.

Extremely high values of specific radioactive sodium content were also found in the stomach. These, too, mostly occurred in the sodium-low animals.

The specific content of the muscle of normal rats remained quite constant over the observed time interval. In the sodium-low animals there was a definite increase, starting at about 200 minutes and continuing for the rest of the 1100 minute interval. This either represents an increase in the extracellular phase or a permeation of sodium into some part of the muscle in which there is not the same rapid exchange of ions as occurs in the interstitial fluid.

The observed accumulation in the skin was quite variable. Toward the end of the time of observation there was a marked reduction in the amount of Na* in the skin in both normal and sodium-low animals.

Apparent Volume of Distribution of Labeled Sodium—The current knowledge concerning the electrolyte and water relations of living tissues has been extensively discussed in reviews (1, 12–14) and original articles (15–19) in recent years. The conception in the main has been that tissues such as muscle consist of an extracellular phase which has approximately the composition of an ultrafiltrate of blood plasma and an intracellular phase which contains no sodium or chloride ions. This concept has been modified by Manery and Hastings (19), who have reached the conclusion that tissues consist of one extracellular phase which is in ionic equilibrium with blood plasma and consists essentially of plasma ultrafiltrate and connective tissue proteins, and two intracellular phases, one of which contains neither sodium nor chloride ions and the other contains chloride and may or may not have sodium in equivalent proportion.

According to these authors, skeletal muscle, heart, liver, spleen, intestine, and brain can be nearly described in terms of the extracellular phase and the first intracellular phase.

The work of Heppel (20, 21) who observed that sodium can replace the depleted muscle potassium of potassium-deprived rats supports the view that sodium can be present in the intracellular phase of tissues.

The data on the specific contents of the labeled sodium given in Table II lend themselves to a calculation of the apparent volume of distribution, or the sodium space of the body and tissues. Similar calculations on the rabbit have been made by Griffiths and Maegraith (10). The data of these authors are not very

accurate because of the very weak radioactive sodium samples available to them.

The assumptions upon which calculations of the relative proportions of the different tissue phases are based are given among others by Hastings and Eichelberger (17) and Manery and Hastings (19). Employing in principle the method of calculation given by Hastings and Eichelberger, we have calculated the apparent volumes of distribution of the labeled sodium and their standard deviations and the results are recorded in Table II.

The following equations were employed in the calculation.

$$(F)_{\mathrm{Na}} = \frac{\{\mathrm{Na}^*\}_T}{\{\mathrm{Na}^*\}_F} \times 100$$

where $(F)_{\mathrm{Na}}$ is the sodium space in per cent of total water, $\{\mathrm{Na}^*\}_T$ the specific Na* content per gm. of tissue water, and $\{\mathrm{Na}^*\}_F$ the hypothetical specific Na* content of the sodium space. Now

$$\{\mathrm{Na}^*\}_T = (\mathrm{Na}^*)_T / T_{\mathrm{H_2O}}$$

where $(\mathrm{Na}^*)_T$ is the specific Na* content per gm. of fresh tissue and $T_{\mathrm{H_2O}}$ the water content of the tissue, and

$$\{\mathrm{Na}^*\}_F = \frac{0.95}{0.92} \times (\mathrm{Na}^*)_P$$

in which $(\mathrm{Na}^*)_P$ is the specific Na* content of the blood plasma and the factors 0.95 and 0.92 are the Donnan distribution ratio and water content of the blood plasma, respectively. When these relations are combined, there is obtained the equation

$$(F)_{\mathrm{Na}} = \frac{0.92}{0.95} \times 100 \times \frac{(\mathrm{Na}^*)_T}{(\mathrm{Na}^*)_P \times T_{\mathrm{H_2O}}}$$

The apparent volume of distribution in the body in per cent of body weight is given by the relation

$$F_{\mathrm{Na}} = \frac{\text{administered Na* minus excreted Na*}}{(\mathrm{Na}^*)_P} \times \frac{100}{\text{body weight}}$$

The values for the apparent volumes of distribution shown in Table II are in good agreement with values calculated by others (1, 19) from the total sodium content of the tissues. Since the rate of uptake of sodium by the tissues is very rapid, it would

TABLE II

Summary of Specific Accumulation and Apparent Volume of Distribution of Labeled Sodium

	Whole body	Body water	Blood plasma	Muscle	Skin + hair	Liver	Kidney	Spleen	Testes	Stomach	Small intestine	Large intestine
Control; average weight = 183 ± 33.4 gm.												
Specific accumulation*.....			1.79	0.26	0.46	0.37	0.79	0.32	0.40	1.18	0.59	0.51
Standard deviation.........			0.42	0.05	0.16	0.13	0.25	0.09	0.16	0.63	0.31	0.22
Apparent volume of distribution†..................	24‡	38	92	16	39	24	47	21	23	79	37	34
Standard deviation.........	3.2	5.1		3.0	14	5.4	12.5	3.4	2.5	51.2	13.4	16.2
Low sodium; average weight = 168 ± 24.6 gm.												
Specific accumulation*.....			2.07	0.33	0.51	0.40	1.06	0.40	0.43	0.80	0.50	0.61
Standard deviation.........			0.28	0.12	0.18	0.13	0.43	0.08	0.20	0.34	0.30	0.24
Apparent volume of distribution†..................	23‡	36.5	92	19	38	22	58	23	23	45	31	35
Standard deviation.........	5.1	8.1		6.7	4.7	1.9	26.9	3.3	8.3	15.9	14.7	14.2

* In per cent of total Na^{24} per gm. of fresh weight.
† In per cent of total water.
‡ In per cent of total body weight.

follow that there is little, if any, of the region of sodium distribution that is not readily permeated by the labeled sodium. The sodium of the tissues covered in the present investigation, it would appear, is essentially all extracellular.

The rise, late in the experimental period, of the specific Na* content of the muscle of the sodium-low rats that is seen in Fig. 3 and the observation of Heppel (20) that about an hour is required for the ratio of muscle Na* to serum Na* to become constant in potassium-deprived rats, however, are open to the interpretation that sodium can be present in the intracellular phase. Obviously further experimental evidence is required to resolve this problem.

The apparent volume of distribution determined for the members of the gastrointestinal tract are high, probably because of incomplete absorption of the labeled sodium in some instances, and later, because of reexcretion with the digestive fluids. The stomach is particularly outstanding in its apparently high volume of distribution. The kidneys also show an extremely high sodium distribution space.

No significant differences were observed in the values of the sodium space between the normal and sodium-low animals.

We are indebted to Professor E. O. Lawrence and the staff of the Radiation Laboratory of the University of California for the radioactive sodium used in these experiments.

SUMMARY

1. Radioactive sodium administered to fasted rats as sodium chloride is absorbed from the gastrointestinal tract even more rapidly than is potassium. The absorption probably takes place by a process of diffusion.

2. The course of the excretion of labeled sodium through the kidney follows an exponential curve. As would be expected, animals on a diet low in sodium retained a larger fraction of the administered sodium than those on an adequate sodium intake.

3. The absorbed sodium is very rapidly distributed throughout the fluids and tissues of the body so that a maximum of specific accumulation is already reached in most cases within about 10 minutes. The specific accumulation then persists for many hours at a constant level. No statistically significant differences were

found between the levels of the sodium content of blood and tissues of the control and those of the sodium-low animals.

4. Calculations of the sodium space of the body and tissues of the rat from the values of the labeled sodium contents showed good agreement with calculations made from total sodium content. This indicates that the labeled sodium rapidly permeates to all regions of the animal where sodium is normally present. The one exception to this was the muscles of the sodium-low rats which showed a steady increase in specific sodium content toward the end of the experimental period.

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STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

I. THE PREPARATION OF HEMORRHAGIC CONCENTRATES*

By HAROLD A. CAMPBELL, WILLARD L. ROBERTS, WILLIAM K. SMITH, AND KARL PAUL LINK

(*From the Departments of Biochemistry and Genetics, Wisconsin Agricultural Experiment Station, University of Wisconsin, Madison*)

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The disease of cattle, known in veterinary practice as "sweet clover disease," arises from the eating of improperly cured hay or silage made from the common sweet clovers *Melilotus alba* and *Melilotus officinalis*. Its occurrence was originally observed practically simultaneously in 1921 by Schofield in Canada and Roderick in this country. Schofield (1, 2) published his observations in 1922 and 1924. Subsequently Roderick (3, 4) and Roderick and Schalk (5) recorded the etiology and pathology of the disease in a comprehensive and thorough study made at the North Dakota Agricultural Experiment Station during the interval of 1921–32.

This disease is in a sense without parallel in animal pathology or human medicine (6–8). When cows, sheep, or rabbits are fed spoiled sweet clover hay made from any of the common *Melilotus* varieties, the disease is manifested by a progressive diminution in the clotting power of the blood and resultant hemorrhages which usually become fatal. It was recognized by Schofield (2) that the disease could be controlled in cattle by the withdrawal of the spoiled hay from the diet and by the injection of blood

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serum freshly drawn from normal cattle, provided the hemorrhagic extravasation had not proceeded too far.

Roderick (4, 5) observed that hemorrhage may occur in almost any part of the body, but most frequently in the subcutaneous and intermuscular fasciae. No visible alteration was found in the blood vessels to explain the internal hemorrhagic diathesis, but external hemorrhage may be readily induced by surgical treatment or accidental injuries. The causative agent in the spoiled hay apparently survives the digestive processes, enters the blood stream, and by some mechanism at present unknown, either prevents the formation of prothrombin in the liver, or effects an *inactivation* of formed prothrombin. Recently Quick (9) confirmed the fundamental observation of Roderick that the delay in the coagulation time of the blood is due to a disturbance of the prothrombin, which is generally recognized as one of the essential components of the blood coagulation mechanism (6–8, 10, 11).

Prior to the biochemical studies begun at this Station in 1934, some efforts had been made by others to extract the causative agent from hay known to induce the hemorrhagic condition characteristic of the disease. Schofield (2) attempted to prepare concentrates by extraction with water. The resulting aqueous extracts were fed to six rabbits. Three of them remained normal. In the other three a delay in the clotting time of the blood was observed and in addition it appeared that some destruction of the red blood cells was induced by the aqueous concentrate.

Roderick and Schalk (5) extracted spoiled hay with water and fed the extract daily to a group of rabbits for approximately a month. The clotting power of the blood of these animals was not altered. In another attempt an aqueous extract of spoiled hay was concentrated under reduced pressure (50–55°). The concentrate was fed to rabbits but no pathological response was noted. When the undissolved residue was fed to rabbits, the usual symptoms of the disease resulted, which indicated clearly that the causative agent, as it occurred in the hay, was not water-soluble.

Roderick and Schalk (5) also attempted to extract the causative agent with 95 per cent ethanol, ethyl ether, and 1 per cent acetic acid. None of these attempts was successful. Subsequently Brown, Savage, and Robinson (12) prepared aqueous extracts of

the spoiled hay which were fed to rabbits without any deleterious effects. The extensive experimentation done by Roderick and Schalk with aqueous extracts of spoiled hay and the findings of Brown *et al.* cast some doubt on Schofield's work, which stands alone in its claim that the causative agent, as it occurs in spoiled hay, can be extracted with water. All other attempts to extract the hemorrhagic agent which had come to our attention when the chemical investigations were initiated at this Station were unsuccessful.[1]

Two fundamental issues confronted us when this study was begun. There were no chemical criteria available to establish the presence of the hemorrhagic agent, and a bioassay offered the only available means of testing the physiological activity of preparations and extracts. In the absence of a chemical test complete reliance had to be placed on a determination of the prothrombin level or activity in rabbit blood or plasma.

We soon recognized that extraction of the hemorrhagic agent in a physiologically active form through a simple and direct operation was not feasible. When the clotting time of whole blood was used for the biological assay, it was found that the hemorrhagic agent was insoluble in petroleum ether, ethyl ether, ethanol, acetone, dioxane, water, and aqueous acid. On the other hand, dilute sodium hydroxide effected the solution of the hemorrhagic principle. In 1936–37 one of us developed a scheme for the preparation of active concentrates (13).

With the prothrombin method of Quick (14) for the bioassay, this extraction scheme, which will not be given in detail, yielded concentrates with much higher activity than the original hay. A bluish green ether solution was obtained which upon removal of the ether gave a green residue weighing 0.52 gm. This concentrate showed a physiological activity equivalent to about 75 gm. of the original hay and thus represents a concentration of about 150-fold. Treatment of the blue-green ether solution with the activated carbon known as Nuchar, after addition of 3 volumes of petroleum ether, removed 0.14 gm. of solid material from the solution. This treatment left a colorless inactive solution. Attempts to elute the physiologically active material were unsuccessful. But when the

[1] Private communications to K.P.L. from various investigators who abandoned the work without publishing their findings.

carbon was fed to a rabbit, a reduction in prothrombin activity was noted equivalent to that obtained after 50 gm. of the original spoiled sweet clover hay were fed. This indicated that at least partial elution of the active principle from the carbon was effected by the digestive system of the rabbit.

After repeated trials we have succeeded in separating the hemorrhagic substance *quantitatively* from the spoiled hay. We are now able to produce consistently an active concentrate with a potency approximately 200 times greater than the original spoiled hay.[2] This concentrate is essentially free from fats, waxes, certain pigments, sugars, glycosides, water-soluble polysaccharides, water-soluble acids, amines, alkaloids, water-soluble proteins, and water-soluble decomposition products of chlorophyll.

At this stage of the investigation the chemical nature of the hemorrhagic agent is still unknown. Consequently each step in the fractionation scheme has been developed and controlled separately through prothrombin assays on standardized susceptible rabbits, selected specifically for this study (15).[2] The activity of each fraction was related to the original spoiled hay rather than to the total solids of the individual active fractions. By evaluating each fraction by the prothrombin assay it became possible to include in the scheme only those operations that effect essentially a quantitative separation of the physiologically active agent.

EXPERIMENTAL

The spoiled sweet clover hay used in this work was produced experimentally[3] from *Melilotus alba* by the procedure developed here by Smith and Brink (16). In its color and tobacco-like suffocating odor, the artificially spoiled hay corresponded to the spoiled hays that are encountered in agricultural practice and, as Roderick showed, are usually fatal to cattle ((5) p. 39). The hay was milled so that it would pass through a screen having openings about 0.5 mm. in diameter.

[2] The method employed to assay potent spoiled sweet clover hays and physiologically active concentrates prepared therefrom by evaluating their capacity to reduce the prothrombin level in rabbit plasma will be given in detail in a subsequent paper.

[3] The spoiled hay used by Quick (9) was obtained in October, 1936, from this experimentally produced stock.

Extraction Procedure (See Diagram)

Step 1—The milled hay (3 kilos) is extracted with Skellysolve A (pentane) for 24 hours in a large Soxhlet extractor (Barnstead Still and Sterilizer Company). The extract containing waxes, fats, and other lipoidal material is inactive. The solvent is allowed to evaporate from the extracted hay at room temperatures.

Step 2—The extracted hay[4] is placed in 30 liters of water to which 260 cc. of concentrated HCl have been added. During the steeping for 48 hours at 25–30°, the suspension is shaken occasionally and if necessary concentrated HCl is added to maintain the pH in the range of 1 to 2. After 48 hours the steepwaters are removed by filtration. They are inactive and contain materials soluble in dilute aqueous acid (carbohydrates, acids, proteins, alkaloids, etc.).

Step 3—The wet filter cake from Step 2 is placed in 30 liters of water containing 120 gm. of NaOH and steeped for 48 hours at 25–30°. The mash is shaken occasionally and if necessary concentrated NaOH is added to maintain a pH of 11 to 12. This treatment effects an extensive swelling and disintegration of the sweet clover tissue. The pectins, hemicelluloses, gums, and water-insoluble acids dissolve in the form of sodium salts. If the liquid is separated from the fiber at this point by filtering or centrifuging, the hemorrhagic fraction is found in the alkaline solution. The fiber is inactive. However, the dispersed pectins, gums, and hemicelluloses make the separation of the fiber and insoluble material very difficult and laborious. In practice this laborious and time-consuming separation is avoided through the technique embodied in Step 4.

Step 4—The alkaline solution (without separation from the solid fibrous material) is acidified by adding concentrated HCl until the solution is at pH 3.0. This precipitates the pectins, hemicelluloses, gums, and water-insoluble acids on the suspended fibers which then act as a carrier and greatly facilitate filtration. The acidified suspension should stand several hours to allow the acid to diffuse into the fibrous material before the filtration is made. The filtrate is inactive, while the precipitated material contains the *total* physiological activity of the spoiled sweet clover hay.

[4] 10 gallon glass-lined vessels are used in Steps 2 and 3.

Step 5—The precipitated material collected on the filter in Step 4 is leached with 20 liters of ethanol (90 to 95 per cent) for 48 hours at 25–30°. The ethanolic extract is removed by filtration. This leaching process is repeated three times and the four

Steps in Concentration of Hemorrhagic Agent

Spoiled sweet clover hay

1. Extract with *n*-pentane

Extract (discard) | (Residue)

2. Steep in water 48 hrs., pH 1–2; filter

Filtrate (discard) |

3. Steep in water 48 hrs., pH 11–12
4. Adjust to pH 3.0; filter

Filtrate (discard) | (Residue)

5. Extract with 95% ethanol; filter

Residue (discard) | (Filtrate)

6. Remove EtOH at 30°
7. Dissolve residue in 0.5% NaOH; adjust pH to 3.0; filter

Filtrate (discard) | (Ppt.)

8. Dissolve in methanol; ppt. with ethyl ether; filter

Ppt. (discard) | (Filtrate)

9. Remove solvent; dissolve residue in 0.5% NaOH; adjust pH to 3.0; extract with ethyl ether

Water layer and insoluble ppt. (discard) | Ether solution Activity 200 × original hay (minimum)

ethanolic extracts are combined. They contain the acids, proteins, chlorophyll degradation products, and some lipoidal material soluble in ethanol. The ethanolic extract is active, while the insoluble fibrous material is inactive.

Step 6—The ethanolic extracts are concentrated in a 5 gallon Pfaudler glass-lined vacuum still under reduced pressure at 25–30°.

Step 7—The residue from Step 6 is removed from the still by dissolving it in 0.5 per cent NaOH. The alkaline solution is acidified by adding HCl until pH 3 is reached to precipitate the active material. The flocculent precipitate formed settles on standing for about 12 hours. The clear supernatant liquid is filtered through an asbestos mat on a Buchner funnel. The brownish green precipitate is finally transferred to the filter and sucked dry. The inactive filtrate is discarded.

Step 8—The active brownish green precipitate obtained in Step 7 is removed from the filter, and dissolved in 1 liter of methanol. To this solution are added 3 volumes of ether, which gives a large flocculent precipitate. This precipitate is removed by filtration through a fluted filter paper. The precipitate is dissolved in methanol and again precipitated by adding ether. This treatment gives a large gummy precipitate which is inactive, and a brownish green active solution.

Step 9—The physiologically active solution from Step 8 is concentrated under reduced pressure at 25–30° to remove volatile solvents. The residue is dissolved in about a liter of 0.5 per cent NaOH and is then precipitated in a finely divided condition by acidifying with HCl. The solution should be definitely acid to Congo red (about pH 3). The suspension is shaken with an equal amount of ethyl ether. After the ether layer is separated from the insoluble material and aqueous layer, the solid material is dissolved by adding NaOH, again precipitated, and shaken with ether. The ether layers, which have a high activity, are combined. The insoluble material suspended in the aqueous layer retains some activity. By repeating the extraction procedure of Steps 8 and 9 on this residue, the remaining activity is almost completely obtained in the ether layer.

The ether solution has a blue-green color and contains the ether-soluble, water-insoluble, acidic compounds such as chlorophyll degradation products, fatty acids, phenolic compounds, organic acids, etc. After the ether is removed under reduced pressure, a concentrate (15 to 18 gm.) is obtained which has constant physiological activity. When 0.60 gm. of this concentrate was fed to standardized susceptible rabbits[2] the plasma prothrombin level was reduced to 10 per cent of the normal in 40 to 48 hours. This

represents a 200-fold concentration (minimum) of the hemorrhagic principle.

SUMMARY

1. An extraction and fractionation scheme has been developed for the concentration of the hemorrhagic substance in spoiled sweet clover hay (*Melilotus alba*).

2. When 0.60 gm. of this concentrate is fed to standardized susceptible rabbits, the plasma prothrombin is reduced to 10 per cent of the normal in 40 to 48 hours. This is equivalent to a 200-fold concentration (minimum) of the hemorrhagic agent.

3. The chemical nature of the hemorrhagic agent present in the concentrates is not known at this stage. However, it can be stated that the concentrate is essentially free from the following classes of substances: fats, waxes, certain pigments, sugars, glycosides, water-soluble polysaccharides, water-soluble acids, amines, alkaloids, water-soluble proteins, and water-soluble decomposition products of chlorophyll.

This series of investigations on the hemorrhagic sweet clover disease was undertaken at the suggestion of Dr. R. A. Brink of the Genetics Department, who in 1933 initiated the first trials at this Station to select a non-bitter strain of sweet clover. We are indebted to Dr. Brink and to Dr. E. A. Hollowell of the Office of Forage Crops and Diseases, United States Department of Agriculture, Washington, for their generous counsel and financial assistance throughout the course of this study.

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IODOMETRIC ESTIMATION OF SMALL QUANTITIES OF NITROGEN WITHOUT DISTILLATION

By MILTON LEVY AND ALBERT H. PALMER

(*From the Department of Chemistry, New York University College of Medicine, New York*)

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The method proposed by Rappaport (6) for the estimation of nitrogen is based on the reaction $3NaOBr + 2NH_3 \rightarrow N_2 + 3H_2O + 3NaBr$. We have modified this method in detail while retaining its basic principle. The principal modifications consist of adoption of the digestion method of Koch and McMeekin (2), a more convenient method for neutralizing the digest, and a more stable hypobromite solution. The modified method has been adapted to two ranges of nitrogen and has been used with protein material. The higher range, which we call a micro[1] range, covers 0.5 to 0.05 mg. of N and the lower one, called a decimicro range, covers 0.05 to 0.005 mg. No difficulties should be met in bringing the method to a centimicro scale, 0.005 to 0.0005 mg., if the techniques of Linderstrøm-Lang and Holter (4) are adopted.

Micromethod—Digestion is carried out with 1 ml. of 1:1 H_2SO_4 and 30 per cent H_2O_2 essentially as described by Koch and McMeekin (2). We have found Hengar[2] granules to be excellent antibumping material. After the digest is clear and cool, 10

[1] In view of the many and inconsistent names used to describe various methods for estimation of small quantities of material, we propose a rational system based on the number of millimolecules in the sample estimated. If the number of millimolecules is in the second decimal place, we call the method a micromethod. If the amount is in the third decimal place, we call it a decimicromethod; and if in the fourth, we call it a centimicromethod, following the system of prefixes used in the metric system. Further extension both upwards and downwards is obvious.

[2] Obtained from the Hengar Company, 1833 Chestnut Street, Philadelphia.

drops of saturated potassium persulfate (5) are added and the digestion carried on for a few more minutes. We have found that persulfate completes the oxidation with greater certainty than H_2O_2. It is not used for the whole digestion because of the resultant accumulation of salt when large quantities of organic matter need to be oxidized. After the oxidation is complete, the peroxides must be removed completely. This is done by adding 1 ml. of H_2O to the digest along with a fresh boiling stone and boiling down to fuming again.

After cooling, the digest is diluted with 10 ml. of H_2O, the mixture is cooled by immersion in tap water, and 5 ml. of the neutralizing reagent added and mixed with cooling. The neutralizing reagent consists of 0.5 N $NaBO_2$ and 3.5 N NaOH prepared by dissolving 30.9 gm. of H_3BO_3 in a liter of 4 N NaOH. The reagent is designed to contain insufficient NaOH to neutralize the H_2SO_4 and more than sufficient $NaBO_2$ to make up the deficit. Each new batch of digestion acid and neutralizing reagent should be tested to be sure that the mixture is alkaline to phenolphthalein.

The hypobromite solution is prepared fresh each day by mixing 20 ml. of a solution of 2.5 ml. of Br_2 and 20 gm. of KBr in a liter of water with 50 ml. of 0.4 N $NaBO_2$ prepared from 100 ml. of 4 N NaOH and 24.7 gm. of H_3BO_3 made up to a liter. The reduced alkalinity of this solution as compared with NaOH-Br_2 mixtures slows the change of NaOBr to $NaBrO_3$ which is inactive in the reaction with NH_3. Exactly 5 ml. of the hypobromite are pipetted into the neutralized digest and, after being mixed, the solutions are allowed to stand at least 2 minutes at room temperature.

To estimate the excess NaOBr, approximately 0.5 gm. of solid KI is added and caused to dissolve by shaking. The solution is then acidified by the addition of 1 ml. of 6 N H_2SO_4 and the liberated iodine titrated with standard 0.01 N thiosulfate; 3 drops of 1 per cent starch in 20 per cent NaCl were used near the end-point.

The amount of nitrogen in the sample in mg. is calculated by multiplying the difference between the titer of the unknown and that of a blank by the normality of the thiosulfate and then by $14.01/3 = 4.67$. The blank usually falls between 12 and 14 ml.

Decimicromethod—The method is essentially the same except

that all quantities of solution are reduced to 0.1 of those in the micromethod. The semiautomatic pipettes described by Levy (3) are used. Since it is inconvenient to make these to hold exact predetermined volumes, allowance is made in the concentration of reagents so that each delivers the proper total quantity of material.

Digestion is carried out in 13 × 100 mm. Pyrex test-tubes with 100 c.mm. of 1:1 H_2SO_4. Heating is conveniently carried out by the use of an Al or Cu block with wells 12 mm. deep to hold the tubes essentially as described by Borsook and Dubnoff (1). A sheet metal strip with holes spaced like those in the block and of a size to catch the rim of the test-tubes is convenient. It is mounted on a ring-stand and, by lifting it, all of the tubes are removed from the heat at once for cooling and addition of H_2O_2, etc.

After addition of the acid and samples and small Hengar granules, the tubes are heated in the block until fuming and charring occur. They are removed, cooled, and a small drop of 30 per cent peroxide added to each, then returned to the block, and the heating continued. This is repeated until the digest remains clear after 3 minutes heating. Finally, 2 small drops of saturated persulfate are added and the tubes heated for at least 5 minutes. The ring of condensing H_2SO_4 should rise about 2 cm. above the heating block if the temperature of the block is right. After cooling, a fresh granule is added with 100 c.mm. of H_2O to each tube and the tubes are placed in an oven at about 120° for 30 minutes to destroy peroxides. After this treatment, the digest is diluted with 1 ml. of H_2O and thoroughly mixed, cooled in tap water, and neutralized by addition of the equivalent of 500 c.mm. of the neutralizing reagent described above. If the pipette does not have this exact volume, the reagent is made more concentrated or dilute as necessary to make the final pH alkaline to phenolphthalein. After cooling, 500 c.mm. of the hypobromite described above are added and the tubes kept at room temperature for 2 minutes. Excess hypobromite is determined by adding approximately 0.05 gm. of KI, dissolving, then adding 100 c.mm. of 6 N H_2SO_4, and titrating from a 2 ml. microburette with 0.01 N thiosulfate. Blanks should run between 1.2 and 1.4 ml. The calculations are as above.

All reagents used should, of course, be as nearly NH_3-free as

possible. Special attention to the 6 N H_2SO_4 used is necessary because variable amounts of any NH_3 in it will be oxidized during its addition to the mixture.

The two methods have been tested on standard $(NH_4)_2SO_4$ solutions, egg albumin, and diluted serum. The last two were standardized by the Kjeldahl method. Some of the tests are summarized in Table I.

TABLE I

Accuracy of Iodometric Method with Protein Solutions

	N present	N found, range	Average error	
	mg.	*mg.*	*mg.*	*per cent*
Micromethod	0.061	0.064 -0.059(5)*	0.0015	2.1
	0.244	0.248 -0.240(5)	0.002	0.9
	0.488	0.478 -0.490(5)	0.006	1.2
Decimicro-method	0.0094	0.0100-0.0090(6)	0.0003	3.0
	0.0107	0.0103-0.0101(4)	0.0005	5.0
	0.0214	0.0210-0.0214(15)	0.0006	2.5
	0.0467	0.0470-0.0461(5)	0.00024	0.5

* Number of samples is given in parentheses.

The methods do not require expensive apparatus and are simple and rapid. We have found them convenient for the estimation of small amounts of nitrogen as encountered in young chick embryos. Other workers have found them useful for estimation of N in allergens, in blood filtrates, and in bacteria.

SUMMARY

Methods for the estimation without distillation of N in samples of 0.5 to 0.005 mg. of N as protein have been described based on the reaction of NH_3 with hypobromite.

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RESOLUTION OF *dl*-PHENYLALANINE BY ASYMMETRIC ENZYMATIC SYNTHESIS

BY OTTO K. BEHRENS, DAVID G. DOHERTY, AND MAX BERGMANN

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

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In a previous communication (1) it was reported that acetyl-*dl*-phenylalanylglycine, when treated with aniline in the presence of cysteine-papain, yields acetyl-*l*-phenylalanylglycine anilide, and that the *d* component of the acetylated dipeptide does not undergo a similar synthesis. Recently, this example of antipodal specificity was reinvestigated with the aid of a highly effective papain preparation. Acetyl-*d*-phenylalanylglycine and both the active isomers of carbobenzoxyphenylalanylglycine were prepared and separately treated with aniline in the presence of cysteine-papain. The *d* forms were found to combine with aniline under the influence of the enzyme, although much more slowly than the *l* forms. The different speed of the enzymatic syntheses of the *l*- and *d*-anilides may be utilized for the preparation of pure *l*-phenylalanine and *d*-phenylalanine, with the easily accessible acetyl-*dl*-phenylalanylglycine as a starting material.

The asymmetric course of the reactions discussed above is caused by the asymmetry of the phenylalanine residue. This residue is not directly involved in the coupling reaction, since it is the carboxyl of the glycine residue that undergoes the anilide formation.

In another set of experiments the glycine residue was replaced by the residues of natural asymmetric amino acids. First acetyl-*l*-phenylalanyl-*l*-leucine, as well as acetyl-*d*-phenylalanyl-*l*-leucine, was combined with aniline in the presence of cysteine-papain. In this case the substrate containing the *l*-phenylalanine residue again reacted markedly faster. Acetyl-*l*-phenylalanyl-*l*-leucine

was transformed into its anilide to the extent of 70 per cent in 24 hours, whereas only 34 per cent of acetyl-*d*-phenylalanyl-*l*-leucine was transformed in 48 hours. The corresponding dehydro compound, acetylaminocinnamoyl-*l*-leucine, was transformed into its anilide to the extent of only 10 per cent in 96 hours.

Similarly both acetyl-*l*-phenylalanyl-*l*-glutamic acid and acetyl-*d*-phenylalanyl-*l*-glutamic acid were transformed into their respective anilides by cysteine-papain, although at markedly different rates. 24 per cent of the first compound was transformed in the course of 2½ hours, whereas the latter compound reacted to this extent only after 24 hours.

The compounds acetyl-*l*-phenylalanyl-*l*-proline and acetyl-*d*-phenylalanyl-*l*-proline were also prepared and subjected to the action of aniline in the presence of cysteine-papain. In both cases, no anilide formation took place. It appears that these acylated dipeptides, containing *l*-proline at the carboxyl end, are resistant to the synthesizing action of cysteine-papain.

EXPERIMENTAL

Enzymatic Synthesis of Acetyl-d-Phenylalanylglycine Anilide—The enzymatic synthesis of the corresponding *l* form from acetyl-*dl*-phenylalanylglycine and aniline has been described previously (1). After filtration of the acetyl-*l*-phenylalanylglycine anilide, the filtrate contained acetyl-*d*-phenylalanylglycine. This was isolated in the following manner. The filtrate was acidified to Congo red with diluted H_2SO_4 and evaporated to dryness *in vacuo*. The residue was extracted with absolute ethanol and the solution evaporated. Acetyl-*d*-phenylalanylglycine was obtained by recrystallizing the residue from water. Needles, with a melting point of 159–161°, were obtained.

$C_{13}H_{16}O_4N_2$.	Calculated.	C 59.1, H 6.1, N 10.6
264.3	Found.	" 59.2, " 6.0, " 10.6

$$[\alpha]_D^{25} = -1.9° \text{ (10\% in } CH_3OH)$$

On treatment of acetyl-*d*-phenylalanylglycine with aniline and cysteine-papain at 40° over a period of 5 days, acetyl-*d*-phenylalanylglycine anilide precipitated in a yield of 72 per cent of the theory. After recrystallization the anilide melted at 208–209° and showed $[\alpha]_D^{25} = -21.0°$ under the same conditions under which the antipode had shown $[\alpha]_D^{25} = +21.3°$ (1).

Preparation of l-Phenylalanine and d-Phenylalanine—To a solution of 64 gm. of acetyl-*dl*-phenylalanylglycine in 120 cc. of acetate buffer, pH 5, there were added, at 40°, 49 cc. of aniline, 1.1 liter of water, and a filtered solution of 2.5 gm. of papain and 1 gm. of cysteine hydrochloride in 500 cc. of water. Within 15 minutes white needles of acetyl-*l*-phenylalanylglycine anilide began to form. After 2 hours the precipitate was filtered off and washed with a bicarbonate solution. 35 gm., with a specific rotation of +18.9° and a melting point of 205–207°, were obtained. In order to isolate the *l*-phenylalanine from this precipitate, it was refluxed with 280 cc. of HCl (20 per cent) for 12 hours. At 0° the hydrolysate deposited 15 gm. of *l*-phenylalanine hydrochloride. A further 5 gm. were obtained on concentration. The hydrochloride was dissolved in 90 cc. of warm water, and decolorized with charcoal. Ammonium acetate was added to the filtrate to bring it to pH 5. At 0°, 11.2 gm. of *l*-phenylalanine with a specific rotation of −35.6° were obtained.

The filtrate from the acetyl-*l*-phenylalanylglycine anilide deposited, in 2 days at 40°, 12.2 gm. of an acetylphenylalanylglycine anilide with an $[\alpha]_D = +3.1°$ (in glacial acetic acid), indicating that this was a mixture of the *d*- and the *l*-anilide. This anilide was discarded. The filtrate now contained almost exclusively the *d* form of the original acetylphenylalanylglycine. The solution was evaporated to dryness *in vacuo* and the residue taken up in 250 cc. of HCl (20 per cent) and refluxed for 12 hours. The hydrolysate deposited, at 0°, 14.5 gm. of *d*-phenylalanine hydrochloride from which 8.4 gm. of *d*-phenylalanine with an $[\alpha]_D = +32.3°$ were obtained.

Carbobenzoxy-l-Phenylalanylglycine—14.1 gm. of carbobenzoxy-*l*-phenylalanylglycine ethyl ester (2) were stirred with 30 cc. of ethanol and 28 cc. of 2 N NaOH, until a homogeneous solution resulted. On addition of 28 cc. of 2 N HCl the acylated dipeptide separated out in the form of colorless needles. Yield, 13.0 gm. The material was recrystallized from ethanol by addition of water and dried at 100° *in vacuo* over P_2O_5. M.p., 151–152°.

$C_{19}H_{20}O_5N_2$.	Calculated.	C 64.0, H 5.7, N 7.9
356.4	Found.	" 63.7, " 5.3, " 8.1 (micro-Dumas)

$[\alpha]^{26}_D = -9.6°$ (5% in glacial acetic acid)

Carbobenzoxy-l-Phenylalanylglycine Anilide—To a solution of 0.89 gm. of carbobenzoxy-*l*-phenylalanylglycine in 2.5 cc. of N NaOH there were added 0.5 cc. of aniline, 15 cc. of citrate buffer (pH 5.0), 50 mg. of cysteine hydrochloride, 10 cc. of papain solution, and water to 50 cc. After 3 hours incubation 0.91 gm. of the anilide had precipitated. It was recrystallized from acetic acid with water and then from methanol. M.p., 180°.

$C_{25}H_{25}O_4N_3$.	Calculated.	C 69.6, H 5.8, N 9.7
431.5	Found.	" 69.4, " 5.6, " 10.0

$[\alpha]_D^{26} = +9.3°$ (5% in glacial acetic acid)

Carbobenzoxy-d-Phenylalanylglycine—The ethyl ester of this compound was obtained with carbobenzoxy-*d*-phenylalanine as a starting material in a manner analogous to the preparation of carbobenzoxy-*l*-phenylalanylglycine ester from carbobenzoxy-*l*-phenylalanine (2). M.p., 109–111°.

$C_{21}H_{24}O_5N_2$ (384.4). Calculated, N 7.3; found, N 7.1

By saponification of the ester, the free carbobenzoxy-*d*-phenylalanylglycine was obtained. M.p., 150–151°.

$C_{19}H_{20}O_5N_2$ (356.4). Calculated, N 7.9; found, N 7.7
$[\alpha]_D^{25} = +9.7°$ (in glacial acetic acid)

Carbobenzoxy-d-Phenylalanylglycine Anilide—This substance was prepared in a manner similar to that described for the *l* form. After 3 hours, 0.29 gm. of anilide had precipitated. After recrystallization, the anilide had a melting point of 179°.

$C_{25}H_{25}O_4N_3$.	Calculated.	C 69.6, H 5.8
431.5	Found.	" 69.4, " 6.0

$[\alpha]_D^{27} = -9.4°$ (5% in glacial acetic acid)

Acetyldehydrophenylalanyl-l-Leucine—To a solution of 26.2 gm. of *l*-leucine in 400 cc. of 0.5 N NaOH there were added 37.4 gm. of acetaminocinnamic acid azlactone and 250 cc. of acetone. The mixture was shaken until the azlactone was dissolved, and then allowed to stand overnight. On addition of 200 cc. of N HCl, acetaminocinnamoylleucine precipitated out. It was filtered at 0° and washed on the filter with ice-cold methanol. Reprecipitation from the solution in aqueous sodium bicarbonate with HCl yielded 40 gm. For analysis, the substance was dis-

solved in methanol and crystallized by the addition of water. Rods, melting at 218–219° (decomposition), were obtained.

$C_{17}H_{22}O_4N_2$.	Calculated.	C 64.1, H 6.95, N 8.8
318.4	Found.	" 64.1, " 6.7, " 8.6

Acetyl-l-Phenylalanyl-l-Leucine and Acetyl-d-Phenylalanyl-l-Leucine—15 gm. of acetyldehydrophenylalanyl-*l*-leucine were dissolved in a mixture of 200 cc. of methanol and 3 cc. of glacial acetic acid. After hydrogenation in the presence of palladium black, the filtered solution was evaporated to dryness. The residue was fractionally crystallized from 50 per cent aqueous dioxane, the first fraction consisting of plates and the later ones of rods.

After recrystallization from methanol-water the fractions exhibited the following characteristics.

Acetyl-*l*-phenylalanyl-*l*-leucine, plates, m.p. 191–193°.

$C_{17}H_{24}O_4N_2$.	Calculated.	C 63.7, H 7.55, N 8.7
320.4	Found.	" 63.65, " 7.4, " 8.7

$[\alpha]_D^{29} = -5.4°$ (10% in absolute alcohol)

After another recrystallization $[\alpha]_D$ was $-5.6°$.

Acetyl-*d*-phenylalanyl-*l*-leucine, rods, m.p. 183–184°. Found, C 63.6, H 7.4, N 8.5. $[\alpha]_D^{29} = -8.3°$ (10 per cent in absolute alcohol). After another recrystallization $[\alpha]_D$ was $-8.4°$.

Acetyl-l-Phenylalanyl-l-Leucine Anilide—To a solution of 0.32 gm. of acetyl-*l*-phenylalanyl-*l*-leucine in 1 cc. of N NaOH there were added 4 cc. of citrate buffer, 10 mg. of cysteine hydrochloride, 9 mg. of papain, and 0.11 gm. of aniline. At 40° crystals began to separate within 10 minutes. After 24 hours the anilide that had separated out corresponded to 70 per cent of the theory. Recrystallized from absolute ethanol, it consisted of long thin rods. M.p., 234–235°. For analysis the anilide was dried *in vacuo* at 78° over P_2O_5.

$C_{23}H_{29}O_3N_3$.	Calculated.	C 69.85, H 7.4, N 10.6
395.5	Found.	" 69.9, " 7.46, " 10.6

$[\alpha]_D^{25} = -41.7°$ (4% in glacial acetic acid)

Acetyl-d-Phenylalanyl-l-Leucine Anilide—The preparation of this substance was carried out as described for the *l* form. After 2 days incubation at 40°, 34 per cent of the theoretically possible amount of anilide had separated. It was recrystallized by addi-

tion of water to its alcoholic solution. Long, fine needles were obtained. M.p., 205–206°. The anilide was dried at 78° over P_2O_5 *in vacuo.*

$C_{23}H_{29}O_3N_3$. Calculated. C 69.85, H 7.4, N 10.6
395.5 Found. " 69.8, " 7.2, " 10.6
$[\alpha]_D^{25} = -24.8°$ (4% in glacial acetic acid)

Acetyldehydrophenylalanyl-l-Leucine Anilide—The preparation of this anilide from acetyldehydrophenylalanyl-*l*-leucine and aniline was carried out in a manner analogous to the previously described preparation of acetyl-*l*-phenylalanyl-*l*-leucine anilide. After 4 days incubation at 40°, 10 per cent of the anilide had separated out. From an alcohol-water mixture the anilide crystallized in elongated plates. M.p., 205–206°. For analysis the anilide was dried at 78° *in vacuo* over P_2O_5.

$C_{23}H_{27}O_3N_3$. Calculated. C 70.2, H 6.9, N 10.7
393.5 Found. " 70.2, " 6.9, " 10.6
$[\alpha]_D^{25} = -5.6°$ (4% in glacial acetic acid)

Acetyl-d-Phenylalanyl-l-Glutamic Acid—When acetyldehydrophenylalanyl-*l*-glutamic acid is hydrogenated according to the method of Bergmann, Stern, and Witte (3), acetyl-*l*-phenylalanyl-*l*-glutamic acid can easily be obtained by crystallization. The mother liquor, on evaporation *in vacuo,* delivers crude acetyl-*d*-phenylalanyl-*l*-glutamic acid as an oil which can be crystallized by dissolving it in 4 parts of hot water and subsequent thorough cooling. Yield, about 50 per cent of the weight of the starting material. This preparation still contained some acetyl-*l*-phenylalanyl-*l*-glutamic acid, which could be removed by several recrystallizations. Finally, an acetyl-*d*-phenylalanyl-*l*-glutamic acid was obtained, which showed $[\alpha]_D^{25} = -9.0°$ (10 per cent in methanol).

For the purpose of this investigation it was essential to obtain the acetyl-*d*-phenylalanyl-*l*-glutamic acid completely free from the isomeric *l*-phenylalanine derivative. Therefore, the crude material was purified over the dimethyl ester. The dimethyl ester was prepared either by means of diazomethane (3) or by means of saturated methanol-HCl at 0°. In the latter case, after evaporation *in vacuo,* repetition of the esterification, and evaporation, an oil was obtained. It was dissolved in warm ethyl acetate, and the solution extracted with a bicarbonate

solution and washed with water. On cooling of the ethyl acetate solution, the dimethyl ester crystallized. Yield, 60 per cent of the theory. M.p., 129°. $[\alpha]_D^{25} = -20.7°$ (10 per cent in methanol).

For saponification 5.4 gm. of the dimethyl ester were shaken with 30 cc. of ethanol and 33 cc. of N NaOH until solution was complete. After an additional 15 minutes, 35 cc. of N HCl were added, and the solution was evaporated to about 15 cc. Upon thorough cooling 4.4 gm. of acetyl-*d*-phenylalanyl-*l*-glutamic acid crystallized as plates. They were recrystallized from 4 parts of water and dried over P_2O_5. On heating, the substance begins to soften at 95°, liquefies at about 115°, and decomposes above 240°. The air-dried compound contains ½ molecule of water. For analysis the substance was dried at 78° *in vacuo* over P_2O_5.

$C_{16}H_{20}O_6N_2$.	Calculated.	C 57.1, H 6.0, N 8.3
336.3	Found.	" 57.3, " 6.15, " 8.2

$[\alpha]_D^{25} = -9.1°$ (10% in methanol)

Acetyl-d-Phenylalanyl-l-Glutamic Acid Monoanilide—0.33 gm. of acetyl-*d*-phenylalanyl-*l*-glutamic acid was dissolved in a mixture of 0.5 cc. of N NaOH, 0.2 cc. of aniline, and 5 cc. of citrate buffer. To this solution were added 10 mg. of cysteine hydrochloride, 3 cc. of papain, and water to 10 cc. After 24 hours, 24 per cent of the acid had been precipitated as monoanilide. The precipitate had increased to a total corresponding to 37 per cent of the theory after 48 hours. The anilide was recrystallized from a methanol-water mixture, 1:1, and was dried at 78° over P_2O_5 *in vacuo*. M.p., 232–233°.

$C_{22}H_{25}O_5N_3$.	Calculated.	C 64.2, H 6.1, N 10.2
411.4	Found.	" 64.0, " 6.1, " 10.4

$[\alpha]_D^{24} = -118.1°$ (8% in pyridine)

In an earlier paper from this laboratory (4) a melting point of 231° and $[\alpha]_D^{22} = -113.9°$ were reported for a preparation of this anilide which was obtained in a different manner.

In an analogous experiment carried out with acetyl-*l*-phenylalanyl-*l*-glutamic acid, 24 per cent of anilide was formed in the course of 2½ hours, and 41 per cent after 24 hours.

Acetyldehydrophenylalanyl-l-Proline—To a solution of 23 gm. of proline in 333 cc. of 0.6 N NaOH there were added 37.4 gm. of acetaminocinnamic acid azlactone and 160 cc. of acetone. The mixture was shaken until the azlactone was dissolved, and then

allowed to stand for 3 more hours. Then 200 cc. of N HCl were added, and the acetone was removed by evaporation *in vacuo*. The acetyldehydrophenylalanylproline crystallized during the evaporation and was filtered at 0°. Recrystallization from 40 cc. of alcohol by addition of water gave 34 gm. of plates melting at 140–142°. For analysis, a sample was recrystallized from water and dried at 78° *in vacuo* over P_2O_5.

$C_{16}H_{18}O_4N_2 \cdot \frac{1}{2}H_2O$.	Calculated.	C 61.7, H 6.15, N 9.0
311.3	Found.	" 61.8, " 5.9, " 9.0

Two Stereoisomeric Forms of Acetylphenylalanyl-l-Proline—25 gm. of acetyldehydrophenylalanyl-*l*-proline were dissolved in a mixture of 150 cc. of methanol and 5 cc. of glacial acetic acid. After hydrogenation in the presence of palladium black, the filtered solution was evaporated to dryness. The resulting oily material was dissolved in a mixture of 25 cc. of methanol and 75 cc. of ether, and allowed to stand at 0°. After several days 5.6 gm. of crystalline material had separated. This isomer (Form I) was recrystallized from 10 per cent methanol, yielding short rods that melted at 174–175°. The substance dried at 78° over P_2O_5 still contained 1 mole of water.

$C_{16}H_{20}O_4N_2 \cdot H_2O$.	Calculated.	C 59.6, H 6.9, N 8.7
322.3	Found.	" 59.6, " 6.6, " 8.7

$$[\alpha]_D^{25} = -35.3° \text{ (10\% in methanol)}$$

Recrystallized from absolute alcohol, cubes melting at 142° and containing $\frac{1}{2}$ molecule of alcohol were obtained.

The methanol-ether mother liquor from the above crystallization of Form I was evaporated to dryness and allowed to stand in the cold room with added ether, yielding 13.0 gm. of crystals. This material was recrystallized from absolute alcohol and from water, yielding 5.4 gm. of Form II as prismatic rods, m.p. 186–187°.

$C_{16}H_{20}O_4N_2$.	Calculated.	C 63.1, H 6.6, N 9.2
304.3	Found.	" 62.9, " 6.5, " 9.2

$$[\alpha]_D^{25} = -72.7° \text{ (10\% in methanol)}$$

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AN INJECTION MANOMETER ASSEMBLY FOR THE STUDY OF REACTIONS AT STEADY STATE*

By GORDON MARSH AND LOREN CARLSON

(From the Zoological Laboratories, State University of Iowa, Iowa City)

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During the course of experiments (to be published) correlating the effect of hydrogen peroxide on the respiratory metabolism with its effect upon the inherent E.M.F. of frog skin, it became apparent that the technique of addition of peroxide to the (Warburg) manometer vessel from the side bulb was unsuited to the accurate determination of the relation of respiratory rate to peroxide concentration. Addition from the side bulb produced a reaction whose velocity varied continuously until disappearance of the peroxide; the entire reaction was completed in 5 to 10 minutes; and the amount of peroxide which could be added was sharply limited by the tendency of the skin to decompose it. The obvious advantages of an apparatus which would permit the addition of peroxide at a constant rate over a relatively long time period led to the construction of the manometer vessel and injection assembly described below.

The apparatus is diagrammed in Fig. 1 in which *I* is the face view and *II* the side view; *III* is a bottom view of the manometer flask.[1] The latter is a standard Warburg flask of about 18 cc. capacity with a capillary tube, *Q*, issuing from the bottom at an angle of about 135° with the side bulb, *R*, so that it may be coupled without strain to the injection pipette by means of the thick walled rubber tube, *O*. The 3-way stop-cock, *B*, connects the graduated 1 cc. pipette, *D*, to the air or to the manometer flask, *P*. The pipette is filled and discharged by means of the 1.5 cc. syringe, *F*; the volume delivered is read from the position

* Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

[1] These flasks were made for us by the American Instrument Company.

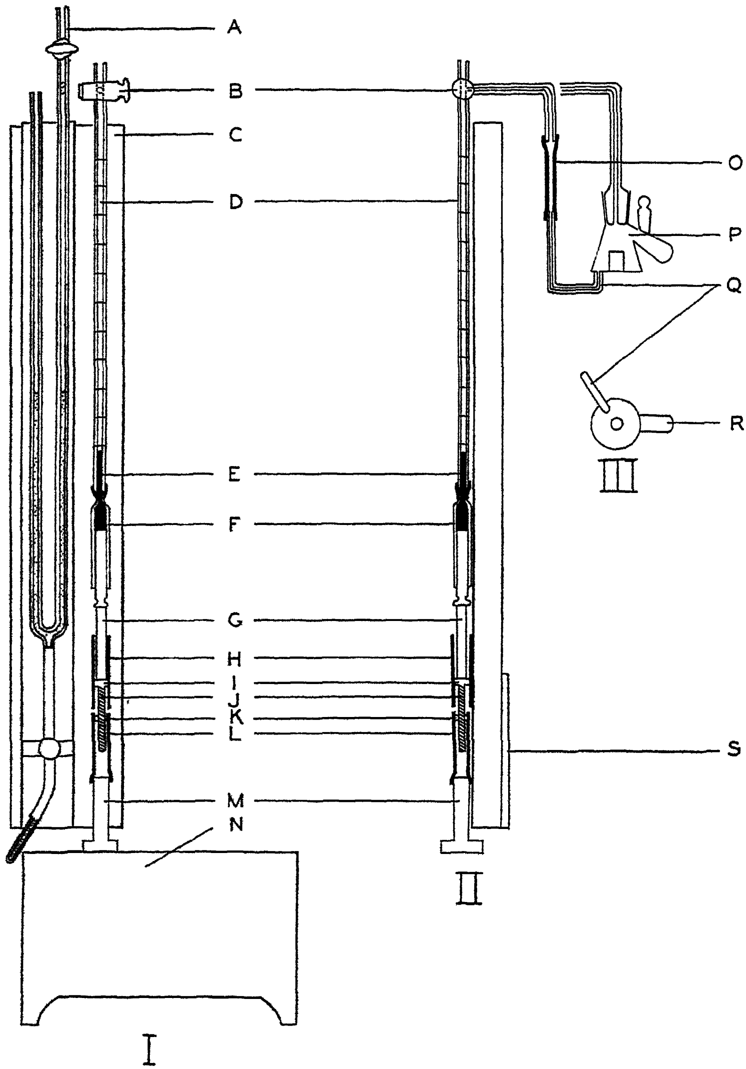

FIG. 1. Injection manometer assembly

of the mercury, *E*. The pipette is actuated by the kymograph, *N*, whose low speed gear rotates the metal shaft, *M*, bearing a

heavy rubber tube, *L*, which has a nut, *K*, seated in the upper end. The nut exerts an upward thrust on the bolt, *J*, which has a pitch of about 10 turns per cm. The bolt head, *I*, is squared to fit the metal guide, *H*. The thrust is communicated to the piston of the syringe by the glass rod, *G*. The support board, *C*, is held to the shaker mechanism (rocking type) by the slotted metal plate, *S*. The slots are in the same vertical line as the injection assembly and the center of oscillation lies at the lower end of the guide, *H*. With the kymograph properly aligned shaking produces no oscillation in the mercury column. The left-hand side of the support board is grooved to hold a standard Warburg manometer, *A*.

Since the fluid volume in the manometer vessel changes continuously during an experiment, the manometer constant must be known for various fluid volumes. The constants are obtained by the standard formula[2] and plotted against V_f. From the plot the average constant for any time interval may be ascertained when the fluid volumes at the beginning and end of the interval are known.

In use the apparatus is made to inject 1 cc. of solution into the flask at a constant rate over a period of 1 hour. By controlling the concentration of the material injected and the speed of the kymograph, both the amount delivered and the time of delivery may be varied within wide limits. The flask is prepared in the usual manner with an initial fluid volume of 2.4 cc., attached to the manometer, and the appropriate gas mixture passed through. The manometer is then placed on the board and connected to the pipette system which has meanwhile been filled. The rubber tube, *O*, is pushed down on the capillary, *Q*, to a predetermined mark at which it is neither stretched nor compressed. This avoids torque on the capillary and prevents slipping of the rubber tube. The volume displaced by the end of the capillary tube is just sufficient to drive the solution to the floor of the manometer vessel. Care must be exercised in filling the pipette and in attaching the tubes to see that no gas bubbles are trapped in the rubber tubing, for if caught at either glass orifice they may undergo a volume change during the ex-

[2] Dixon, M., Manometric methods, Cambridge (1934).

periment which will produce a serious discrepancy between the pipette and the manometer readings. The manometer is then placed in the water bath, equilibrated, a preliminary control run made, the effect of the injection followed, and a final control run made when the injection is finished.

A high degree of accuracy is obtained in the operation of the apparatus. Calibrations of the pipette made by injecting distilled water into the manometer vessel and following the volume change in the manometer can be repeated within the limits of accuracy of reading of the pipette and manometer scales. A steady rate of reaction is reached within a few minutes after the injection is begun. The rate is maintained over an adequate time period, permitting the study of reaction velocities without the complication of rapidly shifting concentrations, an end which cannot be attained by the technique of addition of materials from a manometer side bulb. This offers distinct advantages for the study of the influence on tissue or cell metabolism of metabolites or other materials which are consumed or decomposed. It seems also well adapted to use with enzyme preparations for which information could frequently be more readily gained from the study of steady state conditions than from the analysis of reaction velocity curves.

THE SPECTROPHOTOMETRIC DETERMINATION OF VITAMINS D_2 AND D_3*

By CYRIL H. NIELD, WALTER C. RUSSELL, AND A. ZIMMERLI

(From the Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station and Rutgers University, New Brunswick)

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Many color reactions have been reported for vitamin D, but few (1–5) have been developed for the quantitative determination of this factor. Of the colorimetric methods which had been reported at the time this investigation was begun, the Brockmann-Chen (4) method, which depends on the formation of a yellow color by reaction of the vitamin with a chloroform solution of antimony trichloride, appeared to be the most promising. Although the preparation of an antimony trichloride reagent for the determination of vitamin A involves few difficulties, great care must be taken in its preparation when it is to be used for the determination of vitamin D. Brockmann and Chen (4), Wolff (6), Emmerie and van Eekelen (7), and Ritsert (8) state that the reagent is unstable, its sensitivity changing with time, and that it must be free from alcohol and moisture. Ritsert has found the method exceedingly delicate and claims that reliable results can be obtained by the investigator only after long experience in the preparation and use of the reagent. He reports, furthermore, that difficulty is experienced in preparing reagents of identical sensitivity and that the sensitivity must be redetermined periodically because of the instability of the reagent. Our experience with this reagent fully substantiated Ritsert's claims, and consequently a study of the reaction between antimony trichloride and vitamin D was undertaken in this laboratory with the purpose of developing a method that would give easily reproducible results.

It was thought that the variation in sensitivity might be due to decomposition of the reagent with the formation of acid. It

* Journal series paper of the New Jersey Agricultural Experiment Station, Department of Agricultural Biochemistry.

was found that saturating the antimony trichloride solution with dry hydrogen chloride increased the sensitivity, but the resulting reagent was extremely hygroscopic and difficult to handle. Addition of acetic anhydride eliminated this difficulty but it was effective only over narrow limits of concentration.

Substitution of acetyl chloride for the hydrogen chloride and acetic anhydride eliminated the above disadvantages and produced a reagent which had all the favorable features of the reagent proposed by Brockmann and Chen, exhibited none of its inconsistencies, and was 3 times as sensitive. No difficulty was experienced in reproducing reagents of identical properties. The reagent may be used ½ hour after preparation and is stable for at least 9 weeks. Consequently no periodic control is necessary. A method has been developed with this reagent by means of which it is possible to determine 0.2 γ of vitamin D_2 or D_3.

EXPERIMENTAL

Preparation of Reagent—Merck's reagent chloroform was washed seven times with equal portions of distilled water. The chloroform was shaken with an excess of phosphorus pentoxide and then run rapidly through filter paper. The chloroform was fractionated and the first cloudy portion and the last 10 per cent were discarded. Then 15 to 22 gm. of Merck's reagent antimony trichloride were dissolved in 100 ml. of purified chloroform and the mixture warmed to 35–45° to effect rapid solution of the salt. The solution was filtered, and to every 100 ml. of the filtrate 2.0 ml. of Merck's redistilled acetyl chloride were added.

Spectrophotometric Procedure—Solutions of crystalline vitamins D_2 and D_3 in Merck's reagent chloroform were used in these studies.[1] 0.10 to 1.00 ml. of the test solution containing 2 to 20 γ of vitamin was run from a microburette into a glass-stoppered graduated cylinder, containing enough reagent to make a total volume of 25.00 ml. The optical density of the solution at 500 mμ was determined within 4 minutes in a Bausch and Lomb universal spectrophotometer. The standard cell of the instrument has a capacity of slightly over 23.0 ml.; thus an excess of 2.00 ml. is left for convenience in filling the cell.

In another series of experiments an absorption cell of approxi-

[1] Crystalline vitamins D_2 and D_3 were generously donated by the Winthrop Chemical Company, Inc., Rensselaer, New York.

mately 2.3 ml. capacity was used. It was made by cementing a piece of small bore glass tubing into a 10 cm. polariscope tube. The use of this cell did not impair the accuracy of the method and it permitted the determination of as little as 0.2 γ of vitamin D.

The yellowish pink-colored compound produced by the reagent with vitamin D_2 or D_3 absorbs none of the incident light at 550 mμ and shows maximum absorption at 500 mμ (Fig. 1). This

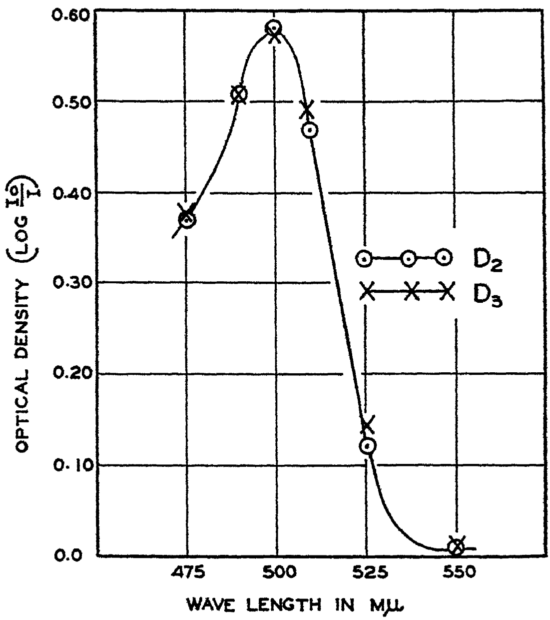

Fig. 1. Absorption curve of the color produced by vitamins D_2 and D_3 with the reagent. The solutions contained 8 γ of vitamin in a total volume of 25 ml.

fact was utilized in determining the optical density of the test solution. The blank cell was eliminated, and the optical density was determined at 500 and 550 mμ. The difference between the two densities represented the absorption due to the reaction product of the vitamin with the reagent. This eliminated errors which might have arisen from differences in general absorption by the blank cell and that containing the test solution.

Absorption Characteristics of Colored Solution—The yellowish pink color reaches its maximum intensity within 30 seconds and

is stable for from 4 to 5 minutes. The average of several readings taken during this period gives an accurate determination of the

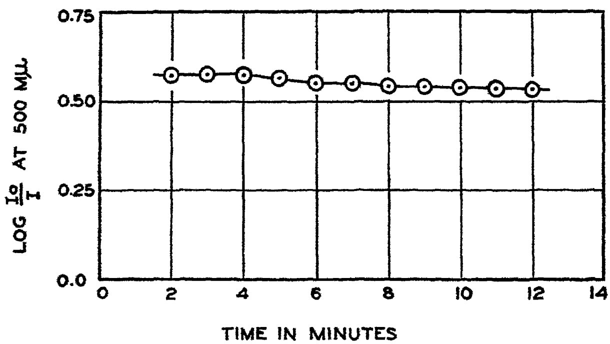

FIG. 2. Time curve of the change of absorption at 500 mμ of the color produced by the reagent with vitamin D_2. The solution contained 8 γ of vitamin in a total volume of 25 ml.

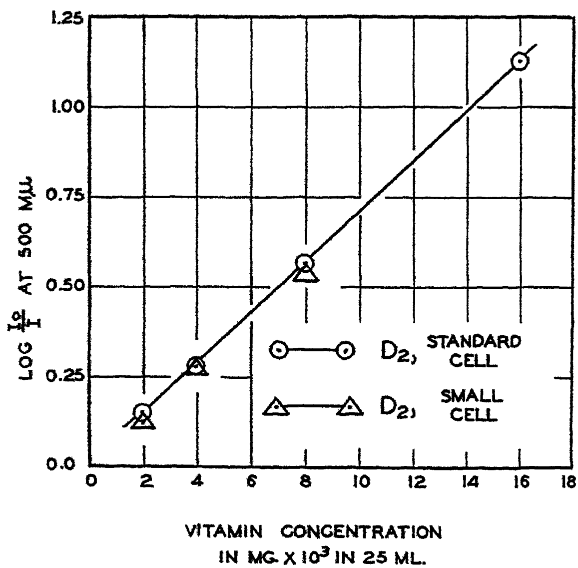

FIG. 3. Relationship between absorption at 500 mμ and the vitamin concentration.

optical density. The color fades slowly, so that after 10 minutes it has lost 7 to 10 per cent of its initial intensity (Fig. 2).

The $E^{1\%}_{1\,\text{cm.}}$ values for crystalline vitamins D_2 and D_3, calculated from the density readings at 500 mμ for 8 γ of vitamin D in a 25 ml. volume (Fig. 1), are of the same magnitude, approximately 1800. Fig. 3 shows that the optical density of the colored solution is proportional to the vitamin concentration. Included in Fig. 3 are three points determined with the small cell of 2.3 ml. capacity, which has been described previously.

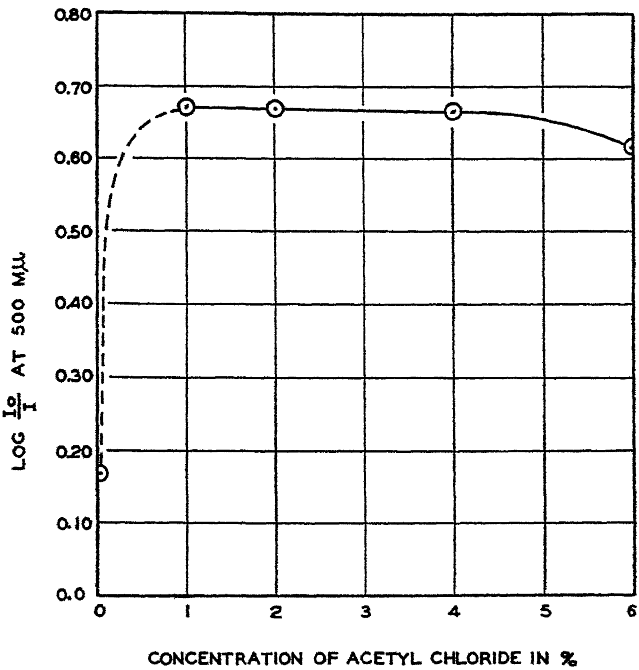

Fig. 4. The effect of acetyl chloride concentration on the sensitivity of the reagent. Points between 0 and 1 per cent were not determined. The solution contained 8 γ of vitamin D_3 in a 25 ml. volume.

Effective Range of Acetyl Chloride Concentration—The limits of acetyl chloride concentration that will yield a reagent of optimum sensitivity are conveniently wide. Fig. 4 shows that reagents containing 1 to 4 per cent acetyl chloride possess the same sensitivity, whereas 6 per cent acetyl chloride diminishes the sensitivity approximately 10 per cent below the optimum, and this concentration is consequently too high.

Effect of Antimony Trichloride Concentration—The concentration of the antimony trichloride may be varied to a considerable extent without effecting any change in the quality of the reagent. The sensitivity remained essentially constant when the antimony trichloride concentration was varied between 15 and 30 gm. of the salt per 100 ml. of chloroform (Table I). When 10 gm. per 100 ml. of chloroform were used, the sensitivity of the reagent was appreciably below optimum and the coloration faded faster than usual, but after standing 1 week the reagent exhibited optimum sensitivity. A concentration of 22 gm. of salt per 100 ml. of chloroform was used in these investigations.

Effect of Alcohol—Ethyl alcohol in a concentration of 0.3 per cent or less does not interfere with the reaction; 0.7 per cent alcohol

TABLE I

Effect of Antimony Trichloride Concentration on Extinction Coefficient of Vitamin D_3

Age of reagent	Antimony trichloride concentration*					
	30	20	15	10	7.5	5
days						
1	1841	1800	1797	1657		1231
8	1781	1797	1834	1800	1700	1441

* The figures refer to the number of gm. of antimony trichloride added to 100 ml. of chloroform.

interferes slightly, whereas 1.4 per cent alcohol markedly lowers the sensitivity of the reagent. Merck's chloroform, which contains 0.7 per cent ethyl alcohol, cannot be used for the preparation of the reagent without the purification described earlier, since its use results in a sensitivity 5 to 7 per cent below optimum. Indications are that this interference is not merely due to the alcohol content of the chloroform.

Attention is now being given to the determination of vitamin D in milk, poultry feeds, and other products.

SUMMARY

1. A new reagent for the determination of vitamins D_2 and D_3, consisting of a solution of antimony trichloride and acetyl chloride in chloroform, has been described.

2. The limits of concentration of antimony trichloride and acetyl chloride, within which the sensitivity of the reagent is constant, have been determined.

3. The reagent produces a yellowish pink color with vitamins D_2 and D_3 which reaches its maximum intensity within 30 seconds and is stable for from 4 to 5 minutes.

4. The absorption curves of the reaction product of the reagent with vitamins D_2 and D_3 have been determined in a Bausch and Lomb spectrophotometer. The two curves are identical, having a maximum at 500 mμ.

5. The $E_{1\,\text{cm.}}^{1\%}$ values at 500 mμ for vitamins D_2 and D_3 are identical and are approximately 1800, which is about 3 times the value given by the reagent proposed by Brockmann and Chen.

6. The optical density, as determined by the difference in absorption at 500 and 550 mμ, is directly proportional to the vitamin concentration.

7. The lower limit of the amount of vitamin that can be accurately determined by the method described is approximately 0.2 γ.

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PHOTOCHEMISTRY OF THE THIAZOLE COMPONENT OF VITAMIN B_1

By FRED M. UBER AND FRANK VERBRUGGE

(From the Biophysical Laboratory of the Department of Physics, University of Missouri, Columbia)

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Photolysis of the pyrimidine component of vitamin B_1 by ultraviolet radiation has been reported in a recent paper by the present writers (1). It was shown that inactivation resulted not only from the breakdown of the pyrimidine ring structure but also from the loss or alteration of those side groups or radicals known to be essential for the biological functioning of the vitamin. Nevertheless, only 1 quantum out of more than 50 was found to be effective in bringing about inactivation. With the thiazole component an analogous quantitative analysis can be made according to the same method, for it has been shown (2, 3) that the fungus, *Phycomyces blakesleeanus*, can develop quite as efficiently when administered equimolecular quantities of the two components of vitamin B_1 as when given the thiamine itself, and hence an assay for either component can be carried out when the concentration of the other is known. In the case of the "thiazole" molecule, it is clear from the work of both Robbins and Kavanagh (4) and Bonner and Erickson (3) that inactivation may mean either some change in the hydrogen of the 2 position or in the 5-hydroxyethyl group rather than a breakdown of the ring itself, as each of the two groups mentioned has been shown to be essential for growth. Since decomposition of the ring structure is reflected in the loss of selective absorption, these two types of photochemical inactivation may be distinguished experimentally.

EXPERIMENTAL

A sample of 4-methyl-5-β-hydroxyethylthiazole, obtained from Merck and Company through the courtesy of George W. Lewis,

and referred to hereafter as simply "thiazole," has been used for all the reported measurements. Absorption data have been obtained with a medium Hilger spectrograph and Spekker photometer, a tungsten steel spark source, Eastman No. 33 plates, and 1 cm. absorption cells. The single broad absorption band around 2510 Å., as shown in the top curve of Fig. 1 for a concentration of 12.5×10^{-5} M, is in agreement with the published results of Ruehle (5) on the basic cleavage product of vitamin B_1 and related thiazole derivatives. Beer's law was found to be obeyed within the limits of error of the measurements for the

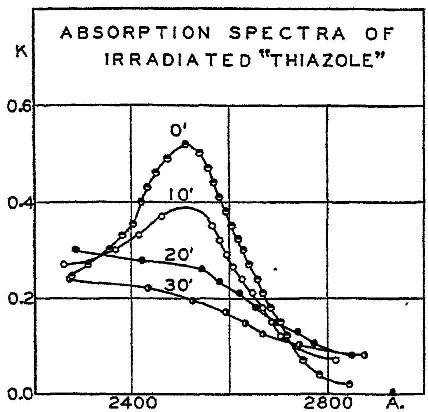

FIG. 1. Absorption spectra of the irradiated "thiazole" component of vitamin B_1. See Table I for values of absorbed energy corresponding to the various exposures.

concentrations upon which the photochemical yields are based.

In our investigation, each liter of nutrient solution contained 100 gm. of dextrose (cerelose), 4.0 gm. of *l*-asparagine, 1.5 gm. of KH_2PO_4, and 0.5 gm. of $MgSO_4 \cdot 7H_2O$ in redistilled water, together with 2-methyl-5-ethoxymethyl-6-aminopyrimidine at a resultant concentration of 1.0×10^{-7} M.

For the control growth curves, the "thiazole" concentration in the nutrient medium was varied from 0.5×10^{-7} M down to zero. After 25 cc. of nutrient solution were added to each of ten 125 cc. Erlenmeyer flasks and sterilized for 12 minutes at 15 pounds pressure, each flask was inoculated with 2 drops of a sterile spore

suspension from cultures of *Phycomyces blakesleeanus*, plus strain, which had been growing for 10 or more days on potato-dextrose-agar slants. The mature cultures in the flasks, having grown for 10 days in a dark oven at 23–24°, were autoclaved and the mycelial mats removed, washed, dried for 24 hours at 95–97°, and weighed.

The control growth curves showing the average dry weight in mg. per culture flask as a function of the "thiazole" concentration for two different determinations are shown in Fig. 2. In the concentration range of 0.025 to 0.250 × 10^{-7} M, the dry weight is

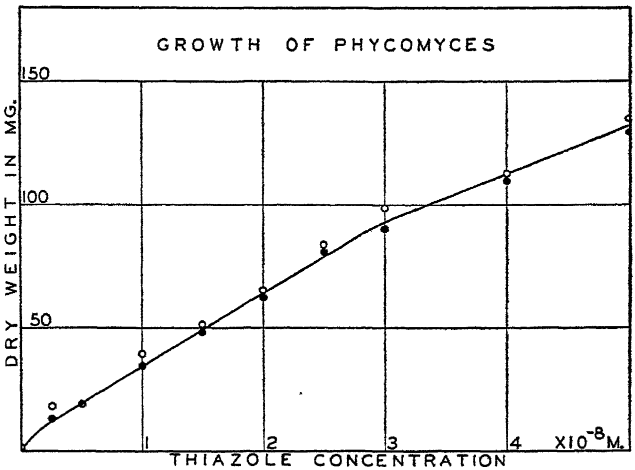

Fig. 2. Increase in dry weight of *Phycomyces* cultures as a function of "thiazole" concentration, with "pyrimidine" concentration constant at 1.0 × 10^{-7} M (two separate trials).

seen to be roughly proportional to the concentration. At still higher concentrations of "thiazole," the growth became limited by the "pyrimidine," whose concentration was maintained constant at 1.0 × 10^{-7} M.

For the photochemical inactivation of the "thiazole," a cylindrical, fused quartz irradiation cell with plane-parallel faces was filled with approximately 5 cc. of a 12.5 × 10^{-5} M solution, buffered with KH_2PO_4 at pH 4.4, and placed at a distance of 10.5 cm. from a low pressure mercury discharge tube (Hanovia Sc-2537), whose ultraviolet emission was almost solely the λ 2537 Å. line. The incident intensity of the radiation was measured with a vacuum

type thermopile, and the absorbing layer of solution, thickness 1.0057 cm., was stirred continuously.

At appropriate intervals during irradiation, a 0.1 cc. sample of the solution was withdrawn from the cell, and its "thiazole" concentration determined by a *Phycomyces* assay based on the culture procedure just outlined and on a comparison with the control growth curves. For this comparison, the growth for any given dose of radiation in the series was computed as a percentage of the growth by a non-irradiated control for a concentration of 0.5×10^{-7} M, made during the same series; a similar calculation was made for the control series data plotted in Fig. 2, and the comparisons were then made on this percentage basis. This procedure seemed justified by the observation that for the concentrations used in the assays, the end-products of irradiation did not inhibit the growth of *Phycomyces*.

Results

During ultraviolet irradiation of the 12.5×10^{-5} M solution of the 4-methyl-5-β-hydroxyethylthiazole, its absorption spectrum undergoes the changes shown in Fig. 1, where the extinction coefficient, K, represents the product of the molecular extinction coefficient and the molar concentration. The energy absorbed by the active "thiazole" solution for the various time intervals indicated on the curves is given in joules per cc. at the top of Column 5 of Table I. A steady decline in the absorption maximum can be noted until finally all selective absorption in this wave-length region disappears, thus signifying the decomposition of the unsaturated thiazole ring structure. But when the extinction coefficients for the inactive decomposition products were plotted as a function of wave-length for various times of irradiation, it was found that they exhibited selective characteristics. This indicates that inactivation has resulted also from alterations in essential radicals as well as from a breakdown of the ring structure. The best guess would seem to be a change in the hydroxyl radical or the entire hydroxyethyl group.

Quantum yields for the inactivation of the essential "thiazole" have been calculated from the four different irradiation trials summarized in Table I. The dry weight in each case is the average for ten culture flasks, with the probable errors as indicated. Column 4 represents the average in per cent of the incident energy

absorbed by the active "thiazole" during the respective irradiation intervals, while Columns 5 and 6 give the total energy absorbed in joules per cc. for various irradiation times and the quanta absorbed per molecule of "thiazole" initially present, respectively. The last column shows the quantum yield at 2537 Å. as determined for each interval separately. The average value is seen to be

TABLE I

Quantum Yield Data for "Thiazole" Inactivation

Exposure time (1)	Dry weight of cultures (2)	Pyrimidine inactivated (3)	Average energy absorbed per interval (4)	Total energy absorbed by active pyrimidine in: Joules per cc. (5)	Quanta per original molecule (6)	Quantum yield (7)
min.	*mg. per flask*	*per cent*	*per cent*			
0	125.4 ± 1.9	00.0		0.000	0.00	
10	77.2 ± 1.1	50.0	53.3	0.079	1.35	0.370
20	46.0 ± 1.0	72.0	31.2	0.125	2.13	0.338
30	19.5 ± 0.4	89.0	17.2	0.151	2.57	0.346
0	125.2 ± 1.3	00.0		0.000	0.00	
10	69.4 ± 1.1	54.2	51.8	0.083	1.41	0.384
20	37.3 ± 0.9	77.5	27.5	0.127	2.16	0.359
30	19.0 ± 0.3	91.5	14.0	0.149	2.54	0.360
0	125.2 ± 1.3	00.0		0.000	0.00	
11	65.0 ± 0.7	57.5	50.8	0.089	1.52	0.378
20	44.2 ± 1.3	72.4	28.7	0.131	2.22	0.326
30	20.2 ± 0.4	90.0	16.8	0.158	2.68	0.336
0	121.8 ± 1.0	00.0		0.000	0.00	
5	97.2 ± 1.1	28.0	60.1	0.048	0.82	0.342
15	59.7 ± 1.1	60.0	42.4	0.116	1.96	0.306
30	21.1 ± 0.8	89.5	21.5	0.167	2.84	0.315
Average............						0.347

0.347, which means that on the average only 1 molecule is inactivated for every 3 quanta absorbed. This may be compared with the value of 0.0184 obtained earlier (1) for the effect of ultraviolet radiation upon the vitamin B_1 component containing the pyrimidine ring. Thus the relative ease with which these two thiamine components are broken down in metabolic processes is reflected in the relative quantum yields for photochemical inactivation.

The above quantum yield has been calculated on the assumption that all the *ultraviolet* radiation from the low pressure mercury discharge tube consists of the single wave-length 2537 Å. As these tubes are known to radiate a few per cent of their energy at λ 1849 Å., where the quantum yield might conceivably be much higher, a check with monochromatic radiation seemed very desirable. An orientation experiment of this kind was performed at λ 2537 Å. with a water-cooled mercury arc and monochromator, but the same yield value was obtained within the limits of experimental error. With the yield so nearly unity, this agreement was anticipated.

SUMMARY

The photochemical decomposition at λ 2537 Å. of the "thiazole" component (4-methyl-5-β-hydroxyethylthiazole) of vitamin B_1 has been demonstrated by its loss of selective absorption and its inability to support the growth of *Phycomyces* cultures.

The quantum yield for inactivation, when inactivation results from changes in side groups as well as from a breakdown of the ring structure, has been found to be 0.347. The corresponding value previously found for the pyrimidine component was 0.0184.

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THE DEPOSITION OF GLYCOGEN WITH WATER IN THE LIVERS OF CATS

By W. O. FENN and LORRAINE F. HAEGE

(*From the Department of Physiology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*)

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In a report recently published it was shown that in rats the deposition of glycogen in the liver is accompanied by such amounts of water, potassium, phosphate, and chloride that the concentration of each of these substances in the liver remains unchanged, while the protein and the acid-insoluble phosphorus become diluted to the extent that would be expected if the original absolute amounts of these substances remained unchanged by the addition of glycogen, water, etc. (Fenn, 1939). This conclusion was not inconsistent with anything in the literature except the experiments of Kaplan and Chaikoff (1936) who concluded that in dogs the glycogen was deposited in the liver without measurable amounts of water.

In order to study this problem further another series of measurements was undertaken with cats in which the liver was analyzed for water, glycogen, protein, total lipid, chloride, and potassium. The animals were fasted or fed plentifully with carbohydrate or treated with phlorhizin in order to vary the glycogen concentration as widely as possible. The results were analyzed graphically and by the method of multiple correlation and were found to confirm in principle the results found on rats. The data of Kaplan and Chaikoff (1936) were analyzed also by the same methods, and it can now be shown that their data also are subject to the same interpretation, and the reasons why the glycogen appeared to be laid down without water have now become clear.

We are much indebted to Dr. Kaplan and Dr. Chaikoff for kindly sending us their complete data and for the courtesy of their correspondence on the subject, as a result of which we seem to be now in essential agreement.

Methods

The cats used in this investigation were taken at random from the animal house and probably had very dissimilar dietary histories. They were anesthetized with an intraperitoneal injection of "dial," 0.65 cc. per kilo. Small liver samples of approximately 0.5 gm. were taken in duplicate from the left medial lobe, blotted on filter paper to remove as much blood as possible, weighed quickly on a torsion balance, and used for the various analyses as described below. Blood samples for plasma chloride analyses were taken from the carotid artery at the end of the sampling procedure which was completed in 8 to 10 minutes.

Glycogen—Because of the rapid breakdown of liver glycogen, livers were routinely sampled first for glycogen. These samples were dropped into tared tubes containing hot 40 per cent KOH. Analyses were carried out according to the methods of Good, Kramer, and Somogyi (1933) and Cori and Cori (1933).

Chloride—Chlorides were determined according to the method described by Manery and Hastings (1939).

Water and Potassium—Water content was determined directly by drying tissue samples in tared weighing bottles to constant weight at 95–100°. The dried samples were subsequently analyzed for potassium by the Shohl and Bennett (1928) method as modified by Fenn and Cobb (1936).

Protein—Wet tissue samples were analyzed for total nitrogen by the Kjeldahl procedure and converted to protein by the factor 6.25.

Total Lipid—A gravimetric method was used for determination of total liver lipid (cholesterol, cholesterol ester, phospholipid, and neutral fat). In principle it was like that described by Kaplan and Chaikoff (1935), but was modified by us to make it applicable to small amounts of tissue (0.5 to 1.0 gm.). Liver samples (of approximately 500 mg.) were weighed on a torsion balance, dropped into beakers containing 95 per cent alcohol and sand (washed in alcohol and ether), and ground to a pulp. These samples were extracted three times with hot alcohol and twice with ethyl ether, being decanted through a filter previously washed in alcohol and ether. The alcohol-ether extracts were pooled, evaporated to approximately 1 to 2 cc., and further extracted with six 5 cc. portions of petroleum ether which were decanted into

tared containers. The gain in weight after the petroleum ether had been carefully evaporated off represented the total lipid extracted from the tissue.

TABLE I

Analyses of Cat Livers after Various Treatments

Cat No.	Weight	Treatment	Liver weight	Glycogen	Protein	Total lipid	Chloride	K	H_2O
	kg.		*gm.*	*per cent*	*per cent*	*per cent*	*mM per kg.*	*mM per kg.*	*per cent*
1	3.3	Sugar*	82.6	8.25	16.44	4.61	32.08	80.6	71.2
2	2.8	Sugar	74.2	9.67	16.70	5.88	30.92	77.2	68.6
3	2.5	"	75.2	8.66	16.77		38.62	81.9	69.6
4	3.3	Normal	99.3	4.66	18.85	6.52	42.4	73.9	71.4
5	2.4	"		2.69	17.44	13.9	40.68	79.4	65.0
6	2.1	"		5.83	18.51	14.3	27.63	72.4	62.4
7	2.8	Sugar*	104.4	12.70	12.56	8.59	34.52	78.8	65.3
8	2.5	Fasting	49.2	4.05	19.12	5.52	39.9	80.4	71.2
9	3.5	"	67.3	2.74	20.32	5.57	43.17	73.1	71.95
10	2.1	"	34.4	3.62	19.40	5.66	36.68	78.0	71.88
11	3.8	"	60.3	2.16	22.40	5.24	37.8	77.5	70.8
12	2.6	Sugar	60.38	6.07	18.26	4.74	36.7	87.5	71.0
13	2.7	"	75.7	1.75	20.10	4.73	30.88	92.3	73.2
14	2.9	"	99.7	9.45	16.50	6.91	32.7	86.5	66.05
15	3.4	Sugar*	98.5	12.64	14.34	5.76	31.5	85.0	66.95
16		"	60.98	6.28	19.36	5.50	39.2	82.9	69.3
17	3.5	Sugar	79.99	9.17	14.78	14.33	33.4	77.7	62.8
18	3.4	Fasting†	83.1	1.53	21.54	7.75	33.42	79.3	70.45
19	3.4	"‡	65.3	0.075	21.35	6.42	29.2	82.3	72.85
20	4.4	"‡	121.1	0.125	21.50	4.23	43.6	77.6	72.6

Normal animals were taken from stock. Fasted animals were without food but not without water for 2 to 3 days previous to sampling. Animals given sugar were previously fasted for 1 to 2 days, except those indicated (*), which had their regular diet. Sugar was given where indicated as a 50 per cent sucrose solution by stomach tube, usually 25 ml. at a time. This was repeated two to six times at intervals.

† Three subcutaneous injections of 1.3 cc. each of 1:1000 adrenalin at 3 hour intervals previous to sampling.

‡ Injected subcutaneously with 0.5 gm. of phlorhizin in 5 ml. of olive oil on the 1st and 2nd days of a 3 day fast.

Results

The results of all our analyses on twenty livers are included in Table I. The livers are numbered in the order in which they were

analyzed without regard to the variations in the treatment. We have included in Table I only the raw data, omitting the innumerable calculations of various sorts which have been made from them. There is little that can be made out from a superficial examination of the figures in Table I except that the glycogen does not fall to very low values even during fasting. It was necessary to resort to repeated injections of adrenalin or injections of phlorhizin in order to obtain livers with very low glycogen contents. Rats, on the other hand, soon consume the carbohydrate in their livers almost completely in fasting.

Calculations—In consideration of our previous results with rats and as a result of preliminary graphic analyses of the data it appeared probable that the water content of the liver depended upon the quantities of glycogen, lipid, and protein, the rôle of the lipid being chiefly that of an inert diluent. Therefore for purposes of analysis it was assumed that the water content of any 100 gm. of fresh liver was given by the following equation

$$W = k_1G + k_2L + k_3P + d \tag{1}$$

where $W, G, L,$ and P are the observed contents in water, glycogen, lipid, and protein respectively, all expressed in gm. per 100 gm. of liver. The difference between the observed and the calculated water content is d, while k_1, k_2, and k_3 are coefficients. The mathematical problem is to determine the values of these three constants (all the data on the twenty livers analyzed are used) which will make the sum of squared differences, d^2, smallest; that is, to obtain the usual least squares fit of calculated water contents to the observed.[1] This may be done as follows:

$$\Sigma d^2 = [\Sigma W - k_1\Sigma G - k_2\Sigma L - k_3\Sigma P]^2 \tag{2}$$

For this sum to be least, its partial derivatives with respect to k_1, k_2, k_3 must each be equal to 0.

$$\frac{\partial \Sigma d^2}{\partial k_1} = -2[\Sigma WG - k_1\Sigma G^2 - k_2\Sigma GL - k_3\Sigma GP] = 0 \tag{3}$$

[1] We are much indebted to Dr. Donald R. Charles of the Department of Biology for introducing us to this method and advising us concerning its application.

$$\frac{\partial \Sigma d^2}{\partial k_2} = -2[\Sigma WL - k_1\Sigma GL - k_2\Sigma L^2 - k_3\Sigma PL] = 0 \tag{4}$$

$$\frac{\partial \Sigma d^2}{\partial k_3} = -2[\Sigma WP - k_1\Sigma GP - k_2\Sigma LP - k_3\Sigma P^2] = 0 \tag{5}$$

From these equations the following was calculated to give the best fit to the observed data for the particular relationship assumed.

$$W = 1.63G - 0.19L + 3.35P \tag{6}$$

The standard errors of these constants have also been calculated according to the method described by Whittaker and Robinson (1929) with the following results, $k_1 = 1.63 \pm 0.303$, $k_2 = 0.19 \pm 0.375$, $k_3 = 3.35 \pm 0.107$. It is evident that the coefficient for lipid does not differ significantly from 0, and it is therefore not inconsistent with the data to conclude that lipid is laid down without water. For the glycogen, however, there is considerably less than one chance in a thousand that glycogen is not deposited with water.

Assuming that the lipid is dry and that $k_2 = 0$, the best fit to the data is obtained by the equation

$$W = 1.57G + 3.29P \tag{7}$$

as calculated by the multiple correlation method. These constants will be used in the graphical presentation of our data in order to avoid making an unjustified correction for lipid water.

Through the courtesy of Dr. Kaplan and Dr. Chaikoff we have been provided with all the figures for protein, lipid, glycogen, and water which they obtained on the livers of the thirty-eight dogs of their series. We have analyzed these data by the method outlined above and arrived at the following equation,

$$W = 1.46G + 0.125L + 3.57P \tag{8}$$

The standard errors of these constants were found to be $k_1 = 1.46 \pm 0.209$, $k_2 = 0.125 \pm 0.03$, and $k_3 = 3.57 \pm 0.107$. In this case all the constants are significantly different from 0. It is reasonable that the constant for lipids should be more reliable in these data from dogs than in our cat series because in the former

the lipid values varied from 3 to 65 per cent, whereas in the cats the extreme variation was only from 6 to 14 per cent.

From these figures it is evident that the data of Kaplan and Chaikoff as originally published were misinterpreted. They are in fact entirely consistent with our findings on both cats and rats. The reason why a superficial analysis indicated the deposition of glycogen without water is probably due to the large amounts of lipid which were present. Livers high in fat are in general low in glycogen. If even a small amount of water is included with the fat, the loss of water due to removal of glycogen would be obscured.

Graphical Representation—In order to show visually how well these equations fit the observed data we present a series of graphs from our data on cats and from the data of Kaplan and Chaikoff on dogs. In Fig. 1 from our data the water is plotted against the glycogen, both being calculated in per cent of the weight of lipid-free and glycogen-free liver. Under these conditions any water associated with the glycogen serves to increase the calculated water percentage as glycogen increases. If water is proportional only to the protein, then the water content should be constant and the points should fall along the line marked "dry." Instead they fall along the line which represents the theoretical curve given by Equation 7, calculated from the method of multiple correlation. The two equations from which this "theoretical" line is derived are given in Fig. 1. If P is eliminated by combining the first two of these equations, we have the last equation which represents the "theoretical" line of Fig. 1.

According to the first of the equations in Fig. 1 the weight of the lipid-free and glycogen-free liver is considered to be due only to water plus protein. Strictly a third term for "undetermined substances," ash, etc., should be included, but in our data the total weight of the liver is on the average exactly accounted for by the protein, glycogen, lipid, and water, so that the data fit the theory equally well without including this small additional term. (If included, it merely shifts the "theoretical" line down 1 per cent if the undetermined substances are estimated at 1 per cent, etc.) The reason why our total is too high by this small amount presumably depends chiefly upon our use of the total nitrogen as a measure of the protein. It depends in part also upon our use of

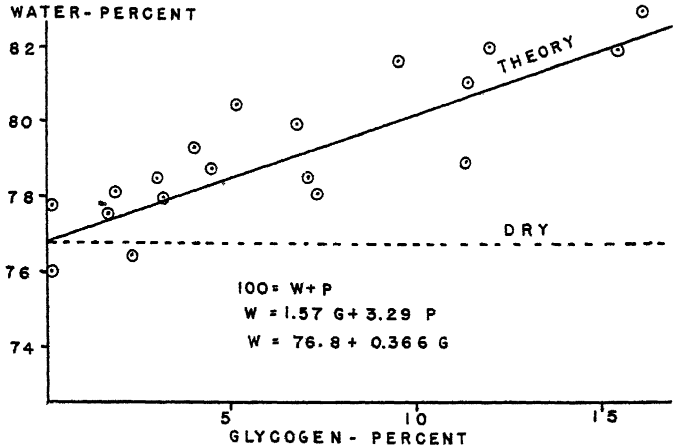

FIG. 1. Water and glycogen in per cent of lipid-free and glycogen-free livers. Data from cat livers.

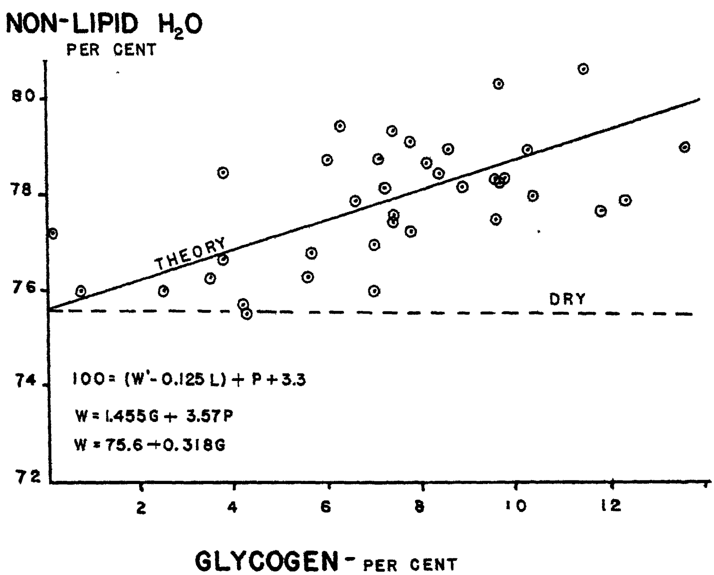

FIG. 2. Non-lipid water and glycogen in per cent of 100 gm. of liver after glycogen, lipid, and lipid water (0.125 gm. of H_2O per gm. of lipid) are deducted. Data of Kaplan and Chaikoff.

total reducing substances instead of fermentable reducing substances as a measure of glycogen. Neither of these minor corrections would change the interpretation of the data significantly.

For comparison with Fig. 1 from our data on cats, Fig. 2 may now be presented from the data of Kaplan and Chaikoff on dogs. The abscissae and ordinates are the same (as in Fig. 1), but in this case the water represents only the non-lipid water and the unit of liver taken as 100 per cent represents the total liver minus lipid, minus glycogen, and minus lipid water which is estimated as 0.125 of the weight of the lipid. Undetermined substances are also included at an estimated value of 3.3 per cent.[2] When the lipid water is deducted, the remaining water is represented by the equation $W = 1.46G + 3.57P$, as calculated from the multiple correlation method. Eliminating P as before, we obtain the theoretical equation represented. Again the points fall better along the theoretical line than around the line representing dry glycogen.

Next we present Figs. 3 and 4 from our data and from those of Kaplan and Chaikoff respectively. Here protein is plotted against glycogen, both being calculated in per cent of the weight of liver after lipid is deducted (and lipid water). In the case of our data it has been assumed that no water was associated with lipid, but the fit would not be appreciably different if 0.125 gm. of water per gm. of lipid were assumed, as in the case of the data from dogs. The addition of glycogen to such a lipid-free liver dilutes the protein so that the protein percentage falls as glycogen increases. If water is included with the glycogen, the amount of dilution for a given amount of glycogen as indicated by the theoretical line is greater than it is for dry glycogen. In both graphs the experimental points follow the theoretical line rather than the dotted line for dry glycogen. The equations involved are given in Figs. 3 and 4 as before, the last equation in each graph being derived from the first two simply by the elimination of W. The last equation is the equation to the theoretical line plotted in each figure.

In Fig. 5 are plotted the potassium contents of the nineteen livers against the glycogen. Both are calculated on the basis of

[2] This value was selected to give the best fit to the data, but the average value from the data was 2.7 per cent, with a variation from 0.9 to 4.5 per cent.

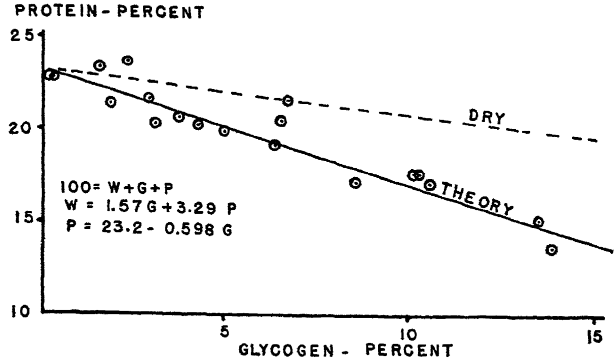

FIG. 3. Protein and glycogen in cat livers in per cent of lipid-free liver.

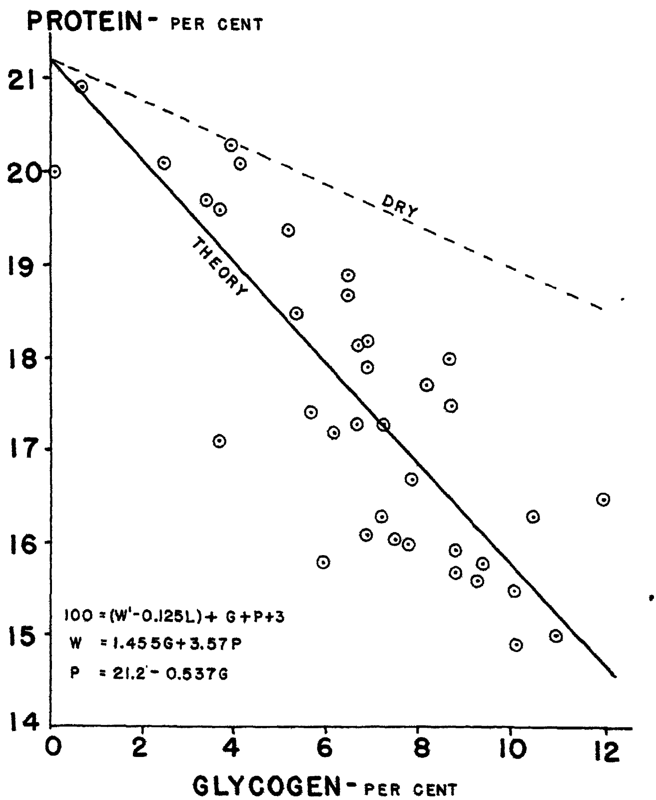

FIG. 4. Protein and glycogen in dog livers in per cent of liver weight after lipid and lipid water (0.125 gm. of H_2O per gm. of lipid) are deducted. Data of Kaplan and Chaikoff.

the lipid-free liver weight. The points have been fitted by the best straight line by the method of least squares and the standard error of the regression coefficient has been calculated. The equation of the line which results is

$$\mathrm{K} = 84.8 + (0.248 \pm 0.217)G \tag{9}$$

There are nearly nine chances out of ten that this slight upward slope is real.

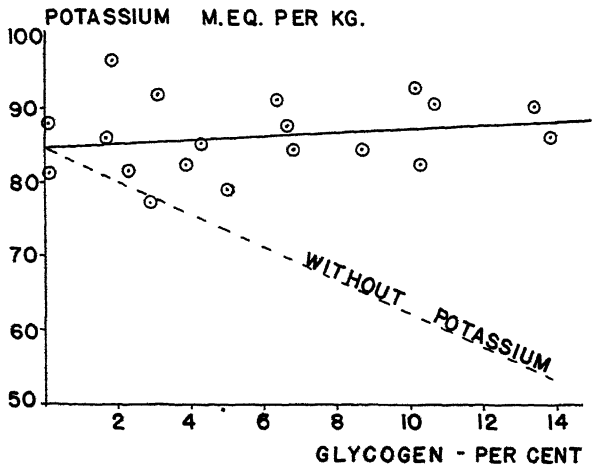

FIG. 5. Showing that potassium accompanies the water which is laid down with glycogen in the liver. The solid line is fitted to the points by the method of least squares. Both potassium and glycogen are calculated on the basis of the lipid-free liver.

Assuming (1) that 1 gm. of glycogen is deposited with 1.63 ± 0.303 gm. of water, (2) that this water is not accompanied by any increase in potassium, and (3) that the potassium content at $G = 0$ is 84.8 milliequivalents per kilo, the theoretical relation between K and G may readily be calculated. This relation is given by the equation

$$\mathrm{K} = 84.8 - (2.23 \pm 0.257)G \tag{10}$$

This equation represents the line marked "without potassium" in Fig. 5. The difference between the regression coefficients in the observed and the theoretical equation is 2.48 ± 0.34, a clearly

significant difference. This is also evident from the graph (Fig. 5), since the experimental points show no tendency to decrease as the glycogen increases.

The slight upward slope of the experimental line in Fig. 5, if real, is probably due to the decreasing chloride spaces of the livers with increasing amounts of glycogen. A larger fraction of such livers is, therefore, intracellular and contains potassium. If the potassium is calculated in milliequivalents per kilo of cell water and if this is plotted against the glycogen (in per cent of liver weight minus chloride space and lipid), the potassium concentration is practically constant. If a straight line is fitted to the points, the equation of the line is

$$\mathrm{K} = 188 + (0.257 \pm 0.71)G \tag{11}$$

Since the standard error of the regression coefficient indicates that it is not significantly different from 0, it is evident that the concentration of potassium in the cell water remains constant within these limits, even though larger amounts of water are added with glycogen.

If the chloride space[3] is plotted against the glycogen (Fig. 6) (both in per cent of the lipid-free liver), a straight line can be fitted to the points by the method of least squares, the equation of the line being

$$\mathrm{Cl\ space} = 31.2 - (0.372 \pm 0.203)G \tag{12}$$

The standard error of the regression coefficient indicates that there is about one chance in thirty that this decrease in chloride space in livers high in glycogen is accidental. In rat livers the chloride space showed no decrease of this sort, and it was concluded that some of the water deposited with glycogen was extracellular and contained chloride in the same concentration as in plasma. In the cat livers this is not so certain, partly because of greater variability in the chloride figures and partly because of the smaller amount of water deposited with glycogen in cats as compared to rats. Assuming (1) that 1 gm. of glycogen is deposited with

[3] The chloride space was calculated as (liver chloride ÷ plasma chloride) × 100 × 0.93 (for plasma water) × 0.96 (Donnan membrane ratio). See Truax (1939) for evidence that chloride space and extracellular space are synonymous in liver.

1.63 ± 0.303 gm. of water, (2) that all the water is intracellular (without chloride), and (3) that the chloride space is 31.2 per cent when $G = 0$, the theoretical chloride space (dotted line, Fig. 6) at different concentrations of glycogen is given by the equation

$$\text{Theoretical chloride space} = 31.2 - (0.82 \pm 0.095)G \qquad (13)$$

The graphs of Equations 12 and 13 are shown in Fig. 6. The difference between the experimental points and the theoretical line is a measure of the amount of chloride deposited with water and glycogen. The difference between the regression coefficients

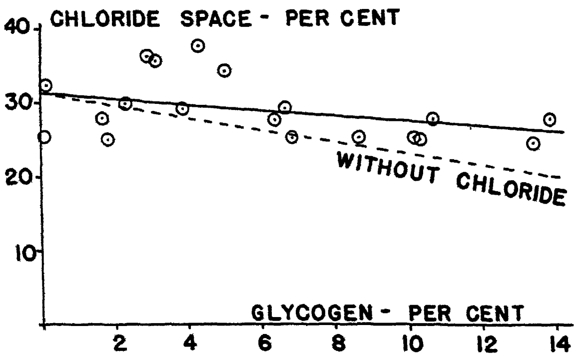

FIG. 6. Showing that some chloride accompanies the water laid down with glycogen (solid line), when the points are fitted by the method of least squares. Both chloride space and glycogen are calculated on the basis of the lipid-free liver.

of the two equations is 0.45 ± 0.22 (standard error). There is, therefore, a probability of about 49 to 1 that this difference is real; *i.e.*, that some of the water deposited with glycogen is extracellular. It is easy to show that the difference means that 0.45 cc. of extracellular water is deposited with each gm. of glycogen added. Since this is only 27 per cent of the total 1.63 cc. of water, while 41 per cent of the total liver water (when $G = 0$) is extracellular, it is evident that the chloride space will be decreased when glycogen is deposited.

From the chloride figures, therefore, it may be calculated that with every gm. of glycogen 1.18 ± 0.22 cc. of intracellular water are deposited. This water should contain potassium and should

serve to increase slightly the potassium content of the liver, as illustrated in Fig. 5. To show how well the chloride and potassium figures agree in this respect it is possible to start with Fig. 5 or Equation 9 and calculate how much intracellular water is needed to explain the potassium figures, assuming in addition (1) that 1 gm. of glycogen is accompanied by 1.63 gm. of total water, (2) that the concentration of K in the intracellular water is 188 milliequivalents per kilo when $G = 0$ (Equation 11). This calculation gives 1.32 ± 0.18 cc. of intracellular water per gm. of glycogen, as compared to 1.18 ± 0.22 cc. from the chloride.

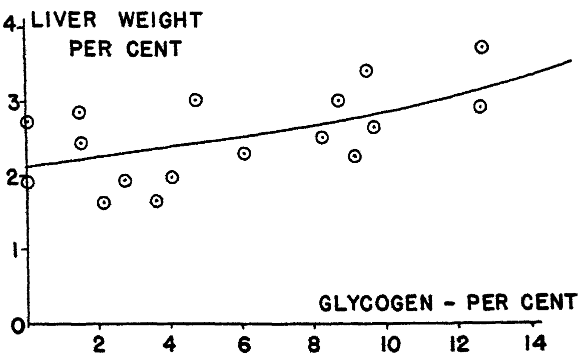

FIG. 7. The liver weight, in per cent of the body weight, tends to increase with glycogen according to the theory that glycogen plus water is simply added to the liver previously present. This theory is represented by the line. The glycogen is expressed in gm. per 100 gm. of fresh weight of liver.

Considering the errors involved, this agreement appears satisfactory.

In our data from rats it was found that the increase in liver weight which accompanied the deposition of glycogen was closely equal to the increase expected if glycogen plus water was added to the liver previously present without replacing any part of it. In the cats this relation is not so definite, probably because of the greater variability of the animals. However, the liver weight in per cent of the body weight does increase with increase in glycogen content and the correlation coefficient between glycogen content and percentage liver weight is +0.79. If we assume as before that 1 gm. of glycogen is laid down with 1.63 gm. of water and

take 2.1 per cent as the percentage liver weight (L) when $G = 0$, the following equation may be derived, giving the theoretical relation between L and G, $L = 211/(100 - 2.63G)$.

The graph of this equation is plotted in Fig. 7 together with the experimental points from Table I. The scatter is large, but there seems to be no systematic deviation from the theoretical curve. It must be concluded, therefore, that the cat livers on the average probably increase in weight in proportion to the added glycogen and water without necessary losses of other substances.

DISCUSSION

In this paper we have presented a formula from which the water content of a cat liver can be calculated if the protein, lipid, and glycogen contents are known. Such a formula is, of course, only an approximation and considers only the three factors which seem to be the most important. Of these the lipid is important chiefly because it is large in amount and inert and "dilutes" the other constituents. Other substances such as electrolytes bear a definite relation to the amount of water present, but it may be supposed that their presence is the secondary result of the amount of water present, although this, of course, is not certain.

The main point which seems to be finally settled by these analyses is that glycogen is definitely associated with appreciable amounts of water when it is laid down in the liver. Presumably this applies also to the deposition of glycogen in muscles. It is not possible to state a universal figure for the amounts of water involved. The figures which have been found per gm. of glycogen are 2.33 cc. in the rat, 1.63 ± 0.303 in the cat, and 1.46 ± 0.209 in the dog.

Apparently from the data of Kaplan and Chaikoff small amounts of water are also associated with the deposition of lipid. This presumably does not apply to all the various fractions of the total lipid, but there is at present no way of discriminating between them in this respect.

SUMMARY

. The livers of twenty cats were analyzed for protein (P), glycogen (G), total lipid (L), water (W), chloride, and potassium. The data were analyzed by the method of multiple correlation, assuming

an equation of the type $W = k_1G + k_2L + k_3P$. The results show that the water content of the liver is best accounted for if 1 gm. of glycogen is deposited with 1.63 ± 0.303 gm. of water, 1 gm. of protein with 3.35 ± 0.107 gm. of water, and lipid without significant amounts of water.

A similar analysis of data previously published by Kaplan and Chaikoff gave a similar result, indicating the deposition of 1 gm. of glycogen with 1.46 ± 0.209 gm. of water, 1 gm. of protein with 3.58 ± 0.107 gm. of water, and 1 gm. of lipid with 0.125 ± 0.03 gm. of water.

Of the 1.63 cc. of water which accompany the glycogen 0.45 ± 0.22 cc. is accompanied by chloride and is therefore probably extracellular. The remainder is accompanied by appropriate amounts of potassium, for the concentration of potassium in the cell water is not diminished by the increase in glycogen. As a result of this distribution of water between cells and interspaces the deposition of glycogen is accompanied by an appropriate small decrease in the chloride content and a corresponding increase in potassium content of the whole liver. The total liver weight increases as its glycogen content increases, as if water and glycogen had been added to what was there previously without replacing any other constituents.

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PURIFICATION OF PROTHROMBIN AND THROMBIN: CHEMICAL PROPERTIES OF PURIFIED PREPARATIONS*

By WALTER H. SEEGERS

(*From the Department of Pathology, State University of Iowa, Iowa City*)

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We wish to describe certain simplifications in the technique previously described (13, 14) for the purification of prothrombin and thrombin. With new adaptations it is possible to process large quantities of plasma and at the same time to obtain products 2 to 5 times as potent as those previously reported from this laboratory. A brief survey of the chemical properties of these newly obtained products is also given.

Preparation of Prothrombin

Beef plasma is diluted 10-fold with water and brought to pH 5.3 with 1 per cent acetic acid. The precipitate is redissolved and $Mg(OH)_2$ suspension is added. In our original technique (14) the prothrombin adsorbed on the $Mg(OH)_2$ was set free with CO_2 at atmospheric pressure. Improvement is achieved by using CO_2 at 4 to 6 atmospheres (by shaking in a metal chamber, with 8 to 10 volumes of water to 1 volume of $Mg(OH)_2$ paste). Following this elution, the CO_2 is allowed to escape, and variable amounts of magnesium carbonate settle out. As previously, the dissolved salts are then removed by dialysis. The use of increased pressure reduces the working volumes at least 90 per cent, and this simplifies enormously the procedure of dialysis.

A second improvement is the isoelectric fractionation of the dialyzed prothrombin-containing eluate; this is accomplished in two steps (at pH 5.6 and at 5.3). The first fraction (pH 5.6)

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usually contains mainly inert protein, whereas the second fraction (pH 5.3) contains much prothrombin of high potency. The latter is particularly potent if small amounts of $Mg(OH)_2$ are used in the adsorption process. If, however, a large excess of $Mg(OH)_2$ is used, there will be marked adsorption of inert protein, most of which will then appear in the first isoelectric precipitate. This voluminous precipitate carries the prothrombin with it, and thus defeats the objective of the fractionation. In order to obtain a combination of good yield and high purity, one should employ intermediate quantities of $Mg(OH)_2$. As an example, 100 cc. of crude prothrombin solution, containing 400 units (17) per cc., should be treated with approximately 15 cc. of 8 per cent $Mg(OH)_2$ suspension.

Preparation of Thrombin

The conversion of prothrombin into thrombin is carried out in a solution of 0.9 per cent NaCl containing 0.15 per cent $Ca(NO_3)_2$. The concentration of prothrombin can be allowed to vary within wide limits, but as a rule the final mixture is made up to contain 2000 to 7000 units of prothrombin per cc.

Thromboplastin, derived from lung, is consumed, apparently in stoichiometric quantities (9) during the formation of thrombin. We make it a practice to use a slight excess over the quantity theoretically required. The thromboplastin used is especially purified (8) to remove traces of antithrombin which are present in crude organ extracts. The pH of the reaction mixture is adjusted to approximately 7.3. It is advisable to add small amounts of imidazole buffer (7).

In such a mixture thrombin formation proceeds rapidly, but usually an hour is allowed for the reaction to come to completion. In order to eliminate electrolytes and to denature some of the protein impurities, the thrombin preparation is now precipitated with acetone, dried with acetone and ether, and then redissolved in water. An electrolyte-free solution can also be obtained by electrodialysis, without the use of acetone. In either case the aqueous solution is brought to pH 5.3 to 5.0 with acetic acid. The precipitate obtained contains inert protein, together with some thrombin carried down by adsorption and coprecipitation. Most of the thrombin, in highly purified form, still remains in solution,

for, unlike prothrombin, thrombin is highly soluble at this pH (see below). The thrombin can be obtained in dried form by acetone precipitation, or by distilling off the water at or below room temperature.

With these improvements in technique the potency is usually 300 units per mg. for prothrombin, and 600 units per mg. for thrombin. The most potent products, 520 and 950 units respectively, are 2 to 5 times as potent as the best previously reported from this laboratory (13, 14). The potency of the thrombin is such that less than 0.006 γ will eventually clot 0.3 cc. of purified fibrinogen solution; and 0.2 γ can be measured conveniently and precisely by our assay technique (17). Normal human plasma contains about 325 units of prothrombin per cc.; hence, its potential thrombin content is less than 37 mg. per 100 cc.

Recently Astrup and Darling (1) have reported work on the purification of thrombin. Their method is similar to our original procedure, except for the omission of the adsorption by $Mg(OH)_2$. These workers report having obtained material with a potency of 10 Mellanby units per mg. 1 Mellanby unit causes clotting of 1 cc. of oxalated plasma in 30 seconds, whereas 1 of our units clots 1 cc. of purified fibrinogen solution in 15 seconds. The inhibiting action of oxalate and plasma antithrombin compensates almost exactly for the difference in the two clotting intervals. The two units are, therefore, almost equivalent, and we conclude that Astrup and Darling's thrombin was only slightly more than 1 per cent as potent as our 950 unit preparations.

Properties of Prothrombin and Thrombin

Solubility—At pH 7.0, prothrombin and thrombin are highly soluble, both in water and in 0.9 per cent NaCl. It is possible to prepare viscous solutions containing 60 per cent or more of the product in question. It is of interest that in aqueous solutions, low concentrations of a variety of salts, including those of Ba, Ca, Sr, and Al (approximately 0.005 M) cause precipitation of prothrombin, but not of thrombin. If the concentration of the salt is increased approximately 5-fold, the prothrombin precipitate redissolves.

When an aqueous solution of prothrombin was acidified with acetic acid, a precipitate appeared at pH 5.6 (Fig. 1). At pH 3.9

the precipitate went completely into solution. In the case of thrombin the precipitation range was pH 5.1 to 3.4. These data give presumptive evidence that the isoelectric point of thrombin is somewhat lower than that of prothrombin.

These same solutions were studied by titration technique to determine how much prothrombin and thrombin actually remained in solution at each pH. Prothrombin activity cannot be followed

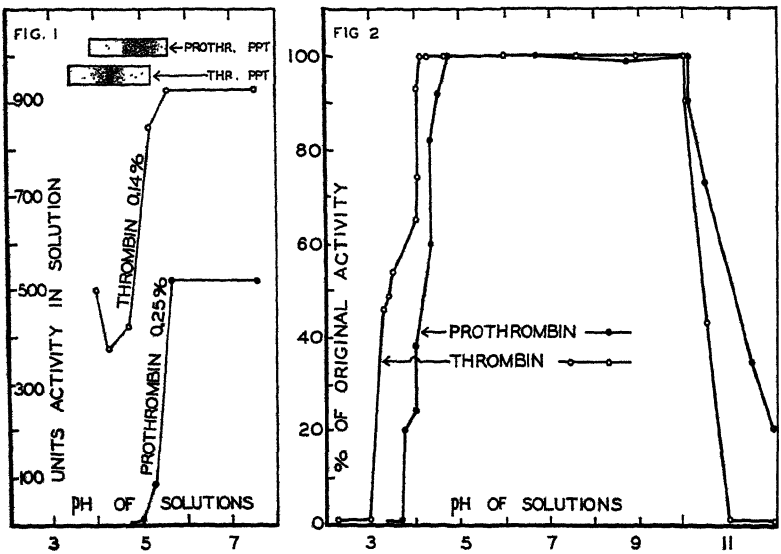

FIG. 1. The solubility of purified prothrombin and of purified thrombin at different pH levels.

FIG. 2. The inactivation of prothrombin and thrombin with acid and with alkali.

below pH 4.7 because of inactivation. However, it is evident from the two curves of Fig. 1 that at their respective points of minimum solubility thrombin is much more soluble than prothrombin. This difference in solubility is a fortunate circumstance, because prothrombin is always accompanied by inert prothrombin-like materials; and, when thrombin is formed, the alteration in solubility and in isoelectric point enables one to eliminate much of this inert material.

Inactivation with Acid and Alkali—Fig. 2 shows the effect of

allowing acid and alkali to act for 30 minutes on saline solutions of prothrombin and thrombin. In the case of thrombin, inactivation began at pH 4.1. At pH 3.5 the inactivation is irreversible but in the range pH 3.5 to 4.1 it is reversible. This can be shown by allowing the activity to regenerate for several hours at pH 7. For example, a solution allowed to stand 30 minutes at pH 3.55 possessed 55 per cent of its original activity the moment it was neutralized. This rose to the 70 per cent level within 2 hours, and to 95 per cent in 4 hours.

In the case of prothrombin, sensitivity to acid is even more marked. Fig. 2 shows that inactivation begins at pH 4.8, and is complete at about 3.5. The problem of reversibility has not been studied as thoroughly as in the case of thrombin. It is of interest that both prothrombin and thrombin are inactivated just on the acid side of their presumptive isoelectric points.

Both prothrombin and thrombin show some inactivation beyond pH 10, and both show marked inactivation beyond the range of pH 11. Fortunately the $Mg(OH)_2$ used as adsorbent in preparing prothrombin has a pH slightly below the beginning of the inactivation zone.

Inactivation by Heating—Heat inactivation studies were made by subjecting aqueous solutions of prothrombin and of thrombin, at pH 7.2, to various temperatures for 30 minutes (Fig. 3). The solutions were then cooled and the thrombin titrated at once (Curve A). The prothrombin solutions were mixed with optimal quantities of calcium and with an excess of thromboplastin. The amount of prothrombin present could then be plotted in terms of thrombin developed (Curve B). Curves A and B show that there was some inactivation of both prothrombin and thrombin at 40°, and at 60° the inactivation was almost complete.

Efforts to restore the activity of the prothrombin were unsuccessful when the product had been heated to 60° or more; but in the range of 40–60° some additional activity could be regenerated by allowing the heated prothrombin to react with calcium and thromboplastin for 30 minutes instead of the usual 10 (see Curve C). Within the zone of 40–60° part or all of the prothrombin is evidently made more sluggish in its reactivity to calcium and thromboplastin. These results correspond rather well with ones reported by Mellanby (5). His inactivation temperatures were

higher, because in his experiments the solutions were allowed to stand for only 5 minutes at the various temperatures.

Dialysis—Neither prothrombin nor thrombin dialyzes through the cellulose acetate membranes which we use (Visking casings). If thrombin solution (1000 units per cc.) is placed on one side of the membrane and plasma on the other, the latter does not clot

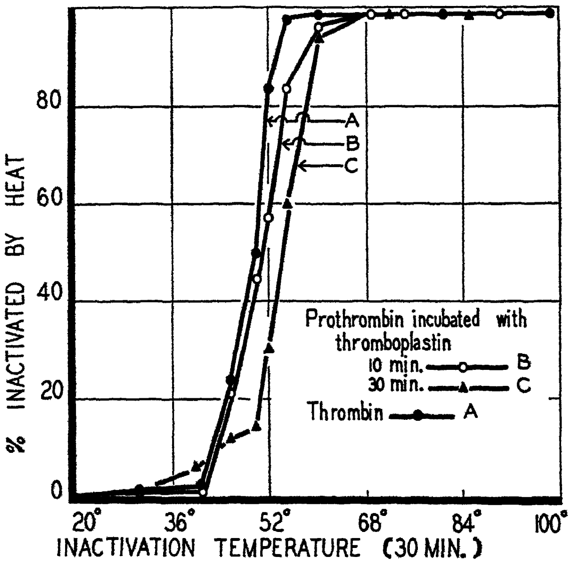

FIG. 3. Approximately 50 unit saline solutions were heated for 30 minutes. One series of prothrombin solutions was allowed to react with optimal quantities of calcium and an excess of thromboplastin for 10 minutes (Curve B), and the other series for 30 minutes (Curve C). With the concentration of thromboplastin used, 10 minutes are sufficient for the complete conversion of unheated prothrombin into thrombin.

within 2 hours. This differs from the results of Mellanby (6), and the discrepancy between his work and ours is probably due to the difference in the quality of the membranes used.

We have undertaken to increase the sensitivity of this test by replacing the oxalated plasma with antithrombin-free fibrinogen solution. Nevertheless, no clot formed in 2 hours. It was possible to show that a solid clot would have formed if 0.01 per cent of the thrombin had passed through the membrane. The failure

of the thrombin to diffuse shows that it is a large molecule, and the analysis reported below indicates that it is a carbohydrate-containing protein.

Chemical Analysis—Elementary analysis, performed by Dr. Carl Tiedcke of New York, showed that the per cent composition of the thrombin product was N 13.23 (Dumas), C 46.37, H 7.35, and ash 3.98. The prothrombin contained N 14.03, C 48.53, H 7.31, and ash 2.03. Sulfur was present in both, but no quantitative determinations were made.

TABLE I

Relative Carbohydrate Content of Some Prothrombin and Thrombin Preparations

Preparation	Activity per mg. N	Carbohydrate content*
	units	*per cent*
Prothrombin 147	1100	3.8
Thrombin 147	4700	5.2
Prothrombin 77	1982	4.7
Thrombin 77	4600	5.1
Prothrombin 155	3620	4.3
Thrombin 155	3210	6.2

* Carbohydrate was estimated by the method of Tillmans and Phillipi (16) as modified by Sørensen and Haugaard (15). The standard was composed of equal quantities of galactose and mannose, and comparisons were made in a colorimeter (12). Glucosamine does not give the color reaction. For that reason these figures may be as much as 35 per cent too low.

The carbohydrate content of the preparations is rather high (Table I), and this helps to account for the fact that the nitrogen content is somewhat lower than for most proteins.

As stated above, when prothrombin is converted into thrombin, there are certain changes in solubility and in apparent isoelectric points which permit one to eliminate certain impurities from the thrombin preparation. The carbohydrate content of the thrombin preparation is thereby increased, indicating that it is associated with the active principle rather than with the impurities. Any remote possibility that the thrombin might be simply a carbohydrate, and the protein present as an impurity, would seem to be minimized by the fact that the activity of our thrombin and prothrombin preparations is readily destroyed by dilute solutions

of HNO_2, at pH 5.5. This evidence would appear to indicate that the activity of the thrombin is dependent upon the integrity of α-amino groups.

Fibrinolysis—A number of workers (2–4, 10, 11) have reported the fact that their thrombin preparations not only cause clotting of fibrinogen, but that eventually they even dissolve the fibrin itself. We have made observations with fibrinogen prepared in a variety of ways, and have found that our purified thrombin preparations give no evidence of fibrinolytic activity over a period of many hours. It would seem likely that the fibrinolytic enzyme is a distinct substance which can be eliminated during the process of purification.

SUMMARY

Prothrombin possessing 300 units of activity per mg. of dry weight and thrombin of 600 units of activity per mg. of dry weight can be obtained routinely by the technique described. Products possessing as much as 520 and 950 units per mg. respectively have been obtained.

Evidence indicates that prothrombin and thrombin are carbohydrate-containing proteins.

Low concentrations of a variety of salts cause precipitation of prothrombin but not of thrombin.

In aqueous solutions prothrombin is relatively insoluble in the neighborhood of pH 4.9. In the case of thrombin the point of minimum solubility is near pH 4.3. When compared at their respective points of minimum solubility, thrombin is much more soluble than prothrombin.

Thrombin, in saline solution, is permanently inactivated by acid at pH 3.5, and reversibly inactivated in the zone pH 3.5 to 4.1. In the case of prothrombin, inactivation begins at pH 4.8, and is complete at pH 3.5.

On addition of alkali, inactivation of both prothrombin and thrombin begins at pH 10 and is quite marked above pH 11.

In aqueous solutions prothrombin and thrombin show partial inactivation after being heated for 30 minutes at 40°. At 60° inactivation is virtually complete.

Nitrous acid destroys the activity of both prothrombin and thrombin.

Our purified thrombin preparations do not show the phenomenon of fibrinolysis.

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A NEW DIETARY ESSENTIAL FOR THE MOUSE

By D. W. WOOLLEY

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

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Our knowledge of the vitamin B complex has been obtained largely by experimentation on the rat and the chick. The use of the dog to demonstrate the essentiality of nicotinic acid and the fact that the vitamin action of pantothenic acid was discovered through the use of the chick have served to emphasize the importance of employing various species in the study of water-soluble vitamins. It is surprising that the mouse has not been widely used for such studies. The rapid growth rate and small food consumption of this species are decided advantages when a basal ration composed entirely of pure chemicals is used. Furthermore, the extensive use of the mouse in numerous types of biological experiments makes an exact insight into its nutritive requirements desirable.

In this paper a dietary essential for the mouse which is necessary for the maintenance of the hair will be described. This factor was discovered during an attempt to show that the mouse requires the additional filtrate factor needed by the rat. Woolley (1) has shown with rats that following the disappearance of the response to additions of pantothenic acid a further gain in weight is produced by certain concentrates. When mice were used in similar experiments, it was observed that many of the animals became hairless. Growth of hair could be restored by suitable dietary supplements.

EXPERIMENTAL

Basal Ration and Assay Technique—The mixture of substances[1] described by Woolley (1) was supplemented with 1 mg. of syn-

[1] Vitamin B_6 was kindly supplied by Merck and Company, Inc.

thetic sodium pantothenate (2). In order to minimize deterioration the ration was used within 1 week from the time of mixing. It was supplied *ad libitum* to weanling male white mice of the Rockefeller Institute strain. These animals weighed 9 to 12 gm. at the start of an experiment. They were kept in individual glass jars with screen bottoms. Each animal was weighed twice weekly. After about 4 weeks on this ration growth had ceased and soon many of the animals had become hairless. However, several water-soluble vitamins were probably absent from the diet.

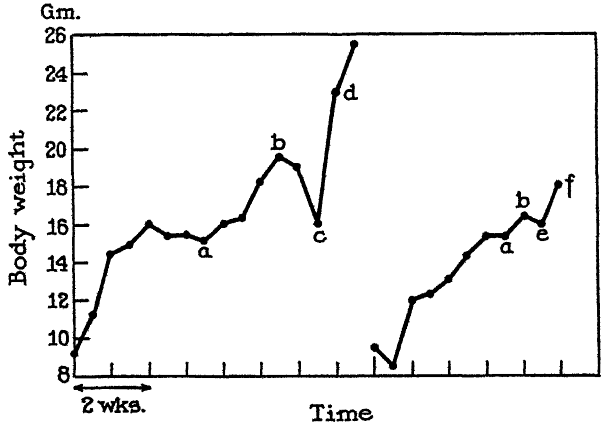

FIG. 1. Typical responses of mice. These two curves represent individual animals in groups started at different times. *a*, added yeast extract; *b*, marked loss of hair; *c*, added alcohol-insoluble fraction of liver extract; *d*, hair restored; *e*, added *p*-aminobenzoic acid; *f*, dead, no restoration of hair.

Preliminary assays revealed that yeast extract was a poor source of the factor curative of alopecia. Therefore this material was used to fortify the ration with some of the unknown vitamins and thus to reduce the possibility of studying a multiple deficiency. When growth had ceased (usually about 4 weeks from the beginning of the experiment), 2 per cent of yeast extract (Difco) was incorporated in the ration. Growth was resumed for a time but soon 50 to 60 per cent of the animals ceased to gain and displayed the symptoms described below. Typical growth curves obtained with animals on the above regimen are shown in Fig. 1.

During the past 7 months, 55 animals have been studied under these conditions.

Description of Symptoms—As the growth response to the addition of yeast extract diminished, the animals assumed an untidy appearance. Abruptly, the hair on the entire body with the exception of the head, tail, and occasionally the legs, fell out, leaving the trunk naked. As the disease progressed, the denuded area became scaly and then quite red. Soon sores appeared. The sore, red, denuded area stood in sharp contrast to the hairy white head and tail. Usually a tuft of hair remained at the base of the tail. The line of demarkation between the hairy head and the hairless body was usually quite sharp. Unless an active concentrate was given, the mice died in 2 to 3 weeks from the onset of symptoms. If an active preparation was given, a normal appearance was presented in 3 days to 1 week. Those substances which did not cause the growth of hair within 10 days were considered inactive.

A small percentage (10 to 20 per cent) of the animals showed evidences of nervous involvement. A few days prior to the loss of hair, an affected mouse appeared hyperirritable and ran around the cage furiously. Soon muscular incoordination was seen, which evidenced itself by erratic movements of hind quarters from side to side. Finally complete paralysis of the hind legs resulted. (It is interesting to note that alopecia first appeared over the hind quarters.) The muscular disorders were apparently not due to lack of the same factor which prevented loss of hair. It was possible to cure the incoordination, and even to restore paralyzed limbs to normal function by feeding a norit eluate of the alcohol-soluble fraction of liver extract.[2] This eluate, however, failed to cause the growth of hair.

Gain in weight has always accompanied restoration of hair with any concentrate thus far tested. However, gain in weight has sometimes been observed with fractions which do not cause growth of hair. For this reason, cure of the symptoms has been used in our work in preference to growth to indicate activity of materials tested. When a fraction appeared to be inactive, the ability of the

[2] This and other liver extracts used in this work were kindly supplied by Dr. David Klein of The Wilson Laboratories.

animals to respond was always tested by administration of an active concentrate.

Sources and Properties of the Factor—Yeast extract and rice bran extract (Vitab)[3] were inactive when fed as 2 per cent of the ration. The 95 per cent alcohol-soluble portion of aqueous liver extract was inactive, except in one instance, when fed as 2 per cent of the ration. However, this material contained some of the active substance, for its use at a 2 per cent level in a prophylactic experiment prevented the loss of hair, and higher levels have been successful in the usual curative tests. The most active source found was the fraction of aqueous liver extract, which was insoluble in 70 per cent alcohol and which had been rendered completely water-soluble by mild enzymatic treatment. 2 per cent of this substance was quite effective (Fig. 1). A similar amount of this fraction was entirely inactive in promoting growth of rats as determined by the method of Woolley (1). Thus it was found that active fractions for the growth of the rat were relatively inactive for the cure of the alopecia in the mouse, and that active mouse fractions were inactive in the rat. These facts indicated that our factor was distinct from the rat growth factors.

The active substance was not readily adsorbed on norit. For example, when 50 gm. of the active liver fraction in 500 cc. of water were stirred with 100 gm. of norit A (Pfanstiehl) it was found that the filtrate was as active as the starting material. It was observed that the active principle was not dialyzable. 100 gm. of the liver fraction in 500 cc. of water were placed in a cellophane tube and dialyzed against running water for 18 hours. No loss of activity was detected in the non-dialyzable portion. It seems probable that the vitamin was non-dialyzable owing to combination with some large molecule, for preliminary tests have indicated that, after the liver fraction had been treated with barium hydroxide and then with lead acetate, the active ingredient was readily dialyzable.

A number of materials of biochemical importance have been assayed. A highly potent biotin concentrate made by the method of Woolley *et al.* (3) was inactive when fed at 10 mg. per 100 gm.

[3] Kindly supplied by Mr. Henry Smith of the Galen Company, Inc.

of ration. *p*-Aminobenzoic acid (4) (2 mg. per 100 gm.) was likewise ineffective. The ether extract of the alcohol-soluble portion of liver extract when fed at a level equivalent to 4 per cent of liver extract was inactive; this material is a good source of the anti-gray hair factor (5, 6).

DISCUSSION

The available evidence indicates that the mouse requires a hitherto undescribed vitamin for normal growth and maintenance of hair. So many vitamins or growth factors have been described that it now has become a major problem merely to demonstrate that a newly discovered syndrome is not due to lack of a vitamin which has already been described. This is particularly true, since many of the newer factors have not been obtained in a state approaching purity. Those vitamins available in crystalline form have been included in our basal ration. Good sources of the unknown rat growth factors were poor sources of our mouse factor and *vice versa*. The involvement of the hair suggested the anti-gray hair factor, but, since an ether extract of liver extract is a good source of this substance, and was ineffective in the cure of alopecia of the mouse, the identity of the two factors seems improbable. The fact that the vitamin existed in liver extract in combination with a large molecule suggested biotin, but direct test of biotin concentrates showed that this growth factor was not effective. Demonstration of relationship to the recently described factors for the chick (7–9) must await purification of these substances.

A simple or descriptive name for this new factor is desirable, and it is proposed that it be called the mouse antialopecia factor.

SUMMARY

When fed a ration of sucrose, purified casein, salts, cod liver oil, corn oil, yeast extract, thiamine, riboflavin, vitamin B_6, nicotinic acid, pantothenic acid, choline, and β-alanine, young mice soon cease to grow, and become completely hairless over the trunk. Hair may be restored by feeding certain fractions of liver extract. The active substance in these fractions is relatively insoluble in alcohol and is non-dialyzable. It has not been identified with any previously described vitamin.

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THE SULFUR DISTRIBUTION IN TOBACCO MOSAIC VIRUS PROTEIN

BY A. FRANK ROSS

(*From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

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The specific properties of many biologically active proteins appear to be dependent upon the presence of sulfhydryl groups or of disulfide linkages. The physiological effect of insulin depends upon the integrity of the latter structure (1, 2), and according to Bersin (3) the disulfide state is also the active one in certain phosphatases, the β-fructosidases, β-glucosidase, and amylase. Free sulfhydryl groups may be essential for the activity of urease (4, 5), papain (5, 6), cerebrosidase (7), lipases (8), and several dehydrogenases (8–11). Sealock and du Vigneaud (12) have shown that the activity of the pressor and oxytocic principles of the posterior pituitary gland depends upon the presence of sulfhydryl or potential sulfhydryl groups. Because of the importance of sulfur in certain proteins and the fact that tobacco mosaic virus contains sulfur (13–15), the nature of its distribution in the virus protein has been investigated preliminary to a study of the rôle which this element may play in virus activity.

It has previously been reported from this laboratory that ultracentrifugally isolated tobacco mosaic virus contains 0.24 per cent sulfur and that the element is not removed by dialysis at pH 9.4 (15). About 60 per cent of the sulfur was accounted for as cysteine or cystine and smaller amounts as methionine and sulfate sulfur. It is the purpose of the present paper to report a more extensive investigation in which it was demonstrated that the content of cysteine plus cystine sulfur in the virus is somewhat larger than that previously reported and that it accounts for practically all of the sulfur. It was also found that the protein

contains little or no methionine, that all of the sulfate sulfur can be removed, and that the total sulfur content of dialyzed protein is approximately 0.20 per cent. There is reason to believe that this sulfur actually occurs in the form of cysteine (16).

EXPERIMENTAL

Preparation of Samples

A chemically isolated sample of tobacco mosaic virus was prepared in several batches from field-grown diseased Turkish tobacco plants (*Nicotiana tabacum*, L.) by use of the isolation procedure of Stanley (17) with trypsin treatment as recommended by Bawden and Pirie (18). The virus was precipitated from the filtered dilute disodium phosphate extract of frozen macerated plants with ammonium sulfate. The precipitate was collected on a celite (Standard) filter pad, dissolved in 0.1 M phosphate buffer at pH 7, and reprecipitated. This procedure was repeated four or five times, after which the protein solution was adjusted to pH 7.5, 0.02 per cent pancreatin was added, and the solution incubated at 38° under toluene for 48 to 72 hours. After two additional precipitations with ammonium sulfate, the protein was precipitated at its isoelectric point by the cautious addition of glacial acetic acid. The precipitate was collected on a celite filter pad and the protein dissolved by suspending the filter cake in water and adding 2 N NaOH dropwise with rapid stirring until the reaction was pH 6.5 to 7. The celite was filtered off and then reextracted three or four times with small amounts of water at about pH 7. The protein solution and extracts were combined, filtered through paper, and then dialyzed until tests showed that less than 0.1 per cent of the total N was ammonia N. The dialyzed solution was concentrated *in vacuo* in an all-glass apparatus (inside temperature 24°) to a thick viscous solution and then frozen and dried *in vacuo*. All operations, except incubation, dialysis, and concentration, were carried out at 5°.

The ultracentrifugally isolated samples were obtained from plants grown in a greenhouse as well as from field-grown plants. The virus was isolated from the filtered extracts from diseased plants by alternate high and low speed centrifugation at 5° (19). The pellets obtained from the fourth ultracentrifugation were transferred to the drying apparatus, frozen, and then dried. The

ultracentrifuged samples were perfectly white, while the chemically isolated sample was a very light gray.

Some of the dried samples were extracted with petroleum ether in an all-glass Soxhlet extractor for 24 hours. Certain of the samples obtained by ultracentrifugation were dialyzed previous to drying in a Kunitz-Simms rocking type dialysis apparatus (20) against flowing distilled water or against a 0.001 M phosphate-citrate-HCl buffer at pH 3 at room temperature. The nucleic acid-free sample was prepared by treating the virus at 0° with 5 per cent NaOH for 1 minute (21). The protein component was precipitated by adding acetic acid until the solution was acid to litmus. The precipitate was removed by centrifugation, then washed with water, and dried. The nucleic acid was precipitated by the addition of an equal volume of alcohol to the supernatant liquid from the protein precipitate after the solution was acidified to Congo red with HCl. The nucleic acid was removed by centrifugation, washed with alcohol, and dried at 110° *in vacuo* over P_2O_5.

Moisture Determinations

Samples dried to constant weight in an oven at 110° or *in vacuo* over P_2O_5 at 37° decreased in weight only slightly upon further drying *in vacuo* over P_2O_5 at 110°. Therefore, samples were dried in an oven at 110° unless otherwise stated.

Total Sulfur Determinations

Total sulfur was determined by the Pregl method by the Arlington Laboratories at Arlington, Virginia. On the basis of analyses on known materials, the analyses reported are regarded as being accurate to within 0.03 per cent; *i.e.*, a sample reported as containing 0.24 per cent sulfur contains not less than 0.21 per cent and not more than 0.27 per cent sulfur. Determinations by means of a Parr bomb were made by Dr. A. Elek on two of the samples, through the courtesy of Dr. P. A. Levene. The results agreed with those obtained by the Pregl procedure.

Colorimetric Cystine Determinations

Hydrolysis—Samples of dried protein or of protein of known moisture content weighing 300 to 600 mg. were hydrolyzed by

heating at 130° with 6 N HCl or at 110–120° with the HCl-HCOOH mixture recommended by Miller and du Vigneaud (22). The hydrolysates were evaporated to a thick gum under a strong current of air and were then dissolved in water.

Humin Formation—Considerable amounts of humin formed in both types of hydrolysates. Some of the samples were decolorized by boiling with norit. The norit was filtered off, washed with hot 0.2 N HCl, and the filtrate and washings diluted to 10 or 25 cc. Other hydrolysates were adjusted to approximately pH 5 with NaOH and the humin removed by centrifugation and then washed with hot water. Aliquots of the decolorized hydrolysates were used for cystine determinations by the Sullivan method (23) and by the Folin-Marenzi procedure (24) or Lugg's modification of the latter (25). The hydrolysates did not give a nitroprusside test; hence the procedures recommended for cystine alone were used.

Methods—The Sullivan method as employed by Miller and du Vigneaud (22) was used, with aliquots containing from 0.25 to 0.40 mg. of cystine. A green filter (Wratten No. 58) was used in the colorimetric measurements.

Application of the Folin-Marenzi procedure gave results that were quite variable and approximately double those obtained by the Sullivan method and often exceeded the amount of sulfur present. Lugg's modification of this method, which introduces a correction for extraneous reducers and provides a rigid control of pH, was therefore used. Determinations were made on aliquots containing 0.30 to 0.50 mg. of cystine.

Results—The results of the colorimetric determination of cystine are given in Table I. A duplicate set of analyses on the chemically isolated virus sample gave comparable results. In addition, a sample was hydrolyzed for 4 days with the HCl-HCOOH mixture and then decolorized at pH 5. Both colorimetric methods gave values of 0.32 to 0.33 per cent. Hence, the two methods gave comparable results only on samples hydrolyzed with HCl for 8 hours or with HCl-HCOOH for 4 days. Following shorter periods of hydrolysis with the latter, the Sullivan method gave the higher results and, in agreement with the findings of Miller and du Vigneaud (22), maximum values were obtained after 48 hours of hydrolysis.

In general, the results were quite variable, did not account for all of the sulfur in the samples, and were considerably lower than values obtained by iodometric titration following hydrolysis with HI. It is probable that the low and variable results were due at least in part to the comparatively large amount of humin formed

Table I

Colorimetric Determination of Cystine in Tobacco Mosaic Virus Hydrolysates*

Description of sample	Method of hydrolysis	Colorimetric method	Method of decolorizing	Cystine*	Cystine* S
				per cent	*per cent*
Ultracentrifugally isolated (0.24% total S)	HCl-HCOOH for 24 hrs.	Sullivan	Norit	0.35	0.09
			pH 5	0.35	0.09
		Folin-Marenzi-Lugg	Norit	0.28	0.07
			pH 5	0.29	0.08
	HCl-HCOOH for 48 hrs.	Sullivan	Norit	0.29	0.08
			pH 5	0.40†	0.11
		Folin-Marenzi-Lugg	Norit	0.24	0.06
			pH 5	0.28	0.07
	6 N HCl for 8 hrs.	Sullivan	Norit	0.36	0.10
			pH 5	0.34	0.09
		Folin-Marenzi-Lugg	Norit	0.31	0.08
			pH 5	0.34	0.09
Nucleic acid-free protein (0.17% total S)	HCl-HCOOH for 48 hrs.	Sullivan	" 5	0.38†	0.10
		Folin-Marenzi-Lugg	" 5	0.52†	0.14

* Calculated as cystine, but would include cysteine.

† Average of three determinations.

during hydrolysis with either HCl or the HCl-HCOOH mixture. Several investigators have reported the loss of cystine or cysteine when boiled with carbohydrates, especially with those that form furfuraldehyde. Dowell and Menaul (26) found that cystine boiled with furfuraldehyde and 10 per cent HCl was deaminated

to the extent of 85 per cent. Bailey (27) reported that the addition of 10 per cent arabinose to edestin before hydrolysis decreased the apparent cystine content from 1.36 to 0.9 per cent when determined by the Folin-Marenzi-Lugg method and from 1.14 per cent to 0.9 per cent when determined by the Sullivan method. Tobacco mosaic virus contains smaller proportions of a furfuraldehyde-forming carbohydrate than this (about 2 per cent pentose) (13, 21), but likewise contains less sulfur than does edestin; hence the effect might be equally great. Lugg (28) found that humin formation results in a considerable loss of cysteine but that cystine is unaffected. It seems likely that at first cysteine was present in the hydrolysates, for a positive nitroprusside test is obtained immediately following denaturation of the virus. Removal of the nucleic acid decreased humin formation and served to increase values given by the Folin-Marenzi-Lugg method but did not alter those obtained by the Sullivan procedure. Recovery of cystine added to the virus protein before hydrolysis was variable and incomplete; hence the colorimetric methods were abandoned and attention was turned to the Baernstein procedure.

Sulfur Distribution after Hydrolysis with HI

Method—Baernstein's (29) method as modified by Kassell and Brand (30) was used, except that the glass-to-glass connection to the first absorber was replaced by rubber tubing and a pinchclamp. As the dried protein samples were very bulky and samples up to 600 mg. were used, it was not practicable to use weighing tubes. Instead, the digestion flask was filled with nitrogen, a weighed sample introduced directly, and the flask immediately connected to the condenser. The average recovery of cystine (3 to 4 mg.) in five determinations was 92.9 per cent; therefore, a correction factor of 107.7 was used in the calculations. The average recovery of methionine (0.8 to 0.9 mg.) as volatile iodide was 90.3 per cent (two determinations) and one determination on 0.34 mg. of sulfate sulfur gave 116 per cent recovery.

The test to establish the identity of the volatile iodide by means of trimethylamine was conducted as described by du Vigneaud, Miller, and Rodden (31). After each run, the contents of Scrubber 3, which consisted of 5 cc. of 3 per cent trimethylamine in absolute alcohol, were sealed in glass, stored at 4°, and examined

TABLE II

Sulfur Distribution in Tobacco Mosaic Virus by Modified Baernstein Method

Description of sample	Preliminary treatment	Total S	No. of determinations	Cysteine*	Cysteine* S	Volatile iodide†	Homocysteine†	Sulfate S
		per cent		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Chemically isolated (from field plants)	Air-dry (corrected for 7.8% H_2O)	0.42	1	0.65	0.17	0.10	0.31	0.05
	Extracted with petroleum ether; dried at 37° *in vacuo* over P_2O_5		3	0.61	0.16	0.12	0.12	0.06
Centrifuged, Sample 1 (from field plants)	Air-dry (corrected for 8.55% H_2O)	0.25	1	0.60	0.16	0.18	0.17	0.04
	Extracted with petroleum ether; dried at 37° *in vacuo* over P_2O_5		3	0.63	0.19	0.14	0.16	0.04
Centrifuged, Sample 2 (from greenhouse plants)	Dried at 37° *in vacuo* over P_2O_5		2	0.73	0.20	0.21	0.16	0.00
	Dialyzed 48 hrs.; dried at 37° *in vacuo* over P_2O_5		2	0.73	0.20	0.11	0.06	0.00
Centrifuged, Sample 3 (from greenhouse plants)	Dried at 37° *in vacuo* over P_2O_5	0.23	1	0.63	0.17	0.11	0.16	0.00
	Dialyzed 48 hrs.; dried at 37° *in vacuo* over P_2O_5		1	0.65	0.18	0.04		0.00
	Dialyzed 96 hrs.; dried at 37° *in vacuo* over P_2O_5	0.19	1	0.70	0.19	0.05	0.16	
	Dialyzed 48 hrs., pH 3.0; dried at 37° *in vacuo* over P_2O_5	0.20	1	0.67	0.18	0.06		
Protein component of tobacco mosaic virus	Dried at 37° *in vacuo* over P_2O_5	0.17	2	0.68‡	0.18‡	0.05‡	0.02‡	0.00

* Calculated as cysteine, but would include cystine.

† Calculated as methionine.

‡ Values corrected for 5 per cent nucleic acid that was removed.

at intervals for the formation of crystals of tetramethylammonium iodide on the walls of the tube.

Results—The corrected values obtained by the modified Baernstein procedure for cysteine, methionine, and sulfate sulfur are given in Table II. The cysteine (or cystine) values were consistently higher than those obtained by the colorimetric methods, and averaged 0.68 per cent, calculated as cysteine, equivalent to 0.18 per cent sulfur. Dialysis or removal of nucleic acid caused no decrease in the cysteine value, whereas the total sulfur content was lowered to about 0.20 per cent (average of four samples). Since this agreement is within the accuracy of the methods, these data provide no basis for assuming that other forms of sulfur are present in tobacco mosaic virus. Analysis of a sample of virus nucleic acid by the same method indicated that not over 3 per cent of the cysteine titration could be attributed to the nucleic acid component. As sulfate was present in some samples and not in others, it must be regarded as a contaminant.

The non-dialysed preparations gave titrations in both the volatile iodide and homocysteine determinations and, since in general the two values agreed, it would appear that methionine was present in such preparations. However, the apparent methionine content was decreased 50 per cent or more by dialysis at pH 3 or against distilled water, treatments which do not lower the activity significantly. The apparent methionine content was also reduced over 50 per cent by removal of nucleic acid. Although the dialyzed or alkali-treated preparations, as well as nucleic acid alone, gave rise to volatile iodide and to homocysteine, the amounts were quite small and their significance doubtful. Extraction with petroleum ether did not decrease the amount of volatile iodide produced. The homocysteine titration is less sensitive and, when so small, little reliance can be placed upon it; hence an attempt was made to determine whether or not the volatile iodide obtained from the virus was actually methyl iodide. In tests with known amounts of methionine, made with the method already described, 0.8 mg. of methionine could be detected and 1.2 mg. resulted in the formation of a large number of tetramethylammonium iodide crystals. However, in one test 3.2 gm. of chemically isolated virus and in another 2.7 gm. of ultracentrifugally isolated virus were hydrolyzed, but in neither case was there any indication of

crystal formation. If the volatile iodide were entirely due to methionine, these samples, according to Table II, should have contained 3.8 and 3.8 mg. of methionine, respectively, of which 1.6 and 1.4 mg., respectively, would not have been removed by dialysis. Since the addition of methyl iodide, equivalent to 1 mg. of methionine, to the 5 cc. of trimethylamine solution used in the tests with protein resulted in the formation of crystals of tetramethylammonium iodide, nothing was evolved during the digestion of the protein that prevented crystal formation. It is evident, therefore, that most of the volatile iodide obtained from non-dialyzed preparations and over half of that from well dialyzed samples could not have been methyl iodide. If the protein contained as much as 0.025 per cent methionine, it would have been detected.

The analyses on the ultracentrifugally isolated samples and the chemically prepared sample are quite similar in all respects except for total sulfur. The sulfur in the former has been largely accounted for, but only about 60 per cent of the sulfur of the latter can be attributed to cysteine and sulfate sulfur. The reason for this is not known.

DISCUSSION

In this investigation no attempt has been made to distinguish between cysteine and cystine, and results are calculated as one or the other as a matter of convenience. The hydrolysates used for the colorimetric methods were treated so that cysteine, if present, would be oxidized to cystine. They gave a negative nitroprusside test. Digestion with HI would, of course, convert any cystine to cysteine. As yet, no free sulfhydryl groups have been demonstrated in the intact virus protein molecule, but they appear following denaturation by even mild means. Titration of the sulfhydryl groups of the virus in 40 to 50 per cent guanidine hydrochloride and in 6 M urea solutions with porphyrindin indicates the presence of 0.76 and 0.70 per cent cysteine, respectively (15). With ferricyanide or tetrathionate in 50 per cent guanidine hydrochloride, a content of approximately 0.65 per cent cysteine was found.[1] The agreement of these values with those found in

[1] Personal communication from Dr. M. L. Anson.

the present investigation clearly indicates that in the denatured protein all or nearly all of the sulfur is in the form of cysteine.

There can be no doubt that the results for cysteine plus cystine obtained by the modified Baernstein method are more accurate than those obtained by colorimetric methods. There is no humin formation during digestion with HI, and the Okuda titration has been shown to be specific for sulfhydryl and disulfide groups (32). Also, the higher value agrees well with the total sulfur and titratable sulfhydryl content of the protein, and there is no reason for suspecting the presence of appreciable quantities of other forms of sulfur.

Since no evidence was obtained that methyl iodide is formed during the hydrolysis of tobacco mosaic virus with HI, and since the homocysteine titration was too small to be of significance, it can be concluded that the virus contains little or no methionine. If as much as 0.025 per cent methionine were present, it would have been detected by the methods that were used. The origin of the volatile iodide is not known. It is interesting to note that du Vigneaud, Miller, and Rodden (31) obtained similar results with insulin.

SUMMARY

A study has been made of the distribution of sulfur in tobacco mosaic virus protein. Iodometric titration following digestion with HI indicated that well dialyzed virus protein isolated by differential centrifugation, or the nucleic acid-free component of the virus, contains 0.68 per cent cysteine or cystine. Since this amount is equivalent to 0.18 per cent sulfur and such protein preparations contain 0.17 to 0.20 per cent total sulfur, all, or nearly all, of the sulfur has been accounted for. This sulfur probably exists in the form of cysteine, since it may be titrated as such in protein denatured by even mild means. Following conversion of cysteine to cystine, colorimetric estimation of the latter by the Sullivan method or Lugg's modification of the Folin-Marenzi procedure after digestion with HCl or a HCl-HCOOH mixture gave lower and less consistent results. A chemically isolated sample of tobacco mosaic virus gave similar results. Traces of sulfate sulfur were present in some samples and absent in others. When the virus was hydrolyzed with HI, a small amount of volatile

iodide was formed and the amount could be reduced by dialysis of the protein. However, it was proved by means of the trimethylamine test that most of the volatile iodide could not have been methyl iodide; hence it seems likely that tobacco mosaic virus does not contain methionine.

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INFLUENCE OF THE EXCRETION OF OTHER PYRIDINE COMPOUNDS UPON THE INTERPRETATION OF THE URINARY NICOTINIC ACID VALUES*

By DANIEL MELNICK,† WILLIAM D. ROBINSON,‡ and HENRY FIELD, Jr.

(*From the Department of Internal Medicine, Medical School, University of Michigan, Ann Arbor*)

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Because of the evidence that nicotinic acid is the pellagra-preventing factor (1), attempts have been made to determine nicotinic acid in blood and urine as methods for estimating the level of nutrition with respect to this essential food factor. A simple procedure for the determination of nicotinic acid in such materials has been published from our laboratory (2). The method gives excellent results with respect to reproducibility of the values and recovery of added nicotinic acid (or amide). However, like all other methods for the determination of this compound, the procedures are not specific for nicotinic acid alone; other pyridine compounds also give the tests. The present report is a study of what other pyridine compounds in the urine react with the reagents (cyanogen bromide and aniline) to give a color which contributes to the final reading as nicotinic acid and how the excretion of such compounds influences the interpretation of the urinary nicotinic acid values. Procedures are also described for converting the nicotinic acid derivatives in the urine, nicotinuric acid and trigonelline, to the free acid, so that some estimation of the extent of excretion of these compounds in the urine can now be made.

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† Upjohn Fellow in Clinical Research, 1937–41.

‡ Upjohn Fellow in Clinical Research, 1938–40.

EXPERIMENTAL

Stability of Nicotinamide in Urine—From each of three normal subjects a 24 hour urine sample was collected with 30 cc. of 3.5 N H_2SO_4 used as a preservative. Half hour aliquots of the acidified

TABLE I

Pyridine Compounds in Urine and Their Reaction with Cyanogen Bromide and Aniline Reagents

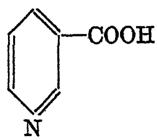
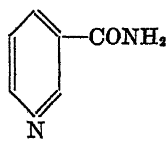
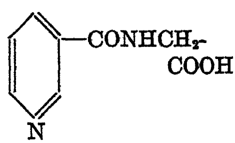
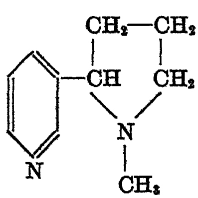
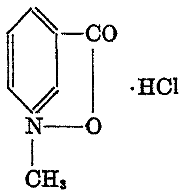
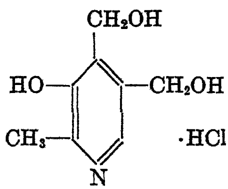
Compound*	Formula	Physical constant (uncorrected)	Quantity tested†	Relative photometric density of reaction product‡
		°C.	γ	
Nicotinic acid§ (3, 4)		M.p., 231	10.0	100
Nicotinamide‖ (3, 4)		" 126	9.9	51
Nicotinuric acid¶ (3, 4)		" 240	14.6	62
Pyridine§		B.p., 115	6.4	88
Nicotine§ (5)		" 116 at 12 mm.	13.2	56

TABLE I—*Concluded*

Compound*	Formula	Physical constant (uncorrected)	Quantity tested†	Relative photometric density of reaction product‡
		°C.	γ	
Trigonelline hydrochloride¶ (3, 4, 6, 7)		M.p., 258	14.1	0
Vitamin B_6 hydrochloride** (8)		" 206	16.7	0

* The numbers in the parentheses after each compound denote references to the earlier investigations demonstrating the presence of these compounds in urine.

† Equivalent on the basis of comparative molecular weights to 10 γ of nicotinic acid.

‡ For the purpose of comparison a value of 100 was assigned to the photometric density of the yellow solution obtained in the test with 10 γ of nicotinic acid.

§ Obtained from the Eastman Kodak Company, Rochester, New York.

|| Furnished by General Biochemicals, Inc., Cleveland, Ohio.

¶ Furnished by Dr. C. A. Elvehjem and Dr. D. V. Frost of the University of Wisconsin.

** Furnished by Merck and Company, Inc., Rahway, New Jersey.

specimens, with 200 γ of nicotinamide added to each, were allowed to stand in stoppered flasks at room temperature for a period of 30 days. Periodically the aliquots were tested (2); in all cases the values remained constant and represented theoretical recoveries of the added amide. Nicotinamide is stable in acidified urine for a period of more than 30 days.

Pyridine Compounds in Urine and Their Reaction with Cyanogen Bromide and Aniline Reagents—In Table I are listed the principal,

known pyridine compounds found in urine. Solutions were made to contain known quantities of these compounds equivalent in each case, on the basis of comparative molecular weights, to 10 γ of nicotinic acid. For the purpose of comparison a value of 100 was assigned to the photometric density of the yellow solution obtained in the test with the 10 γ of nicotinic acid. The results are given in Table I. The difference in reaction between nicotinic acid and the amide is of no consequence, since the hydrolytic procedure used in the application of the reaction to biological materials completely converts the amide to the free acid (2). Nicotinuric acid, the glycine conjugate of nicotinic acid, is the only other compound in the series to possess anti-blacktongue activity (9). This compound likewise can be completely hydrolyzed to yield the free acid (2). Trigonelline, the betaine of nicotinic acid, fails to react with the reagents. Inasmuch as both nicotinuric acid and trigonelline have been isolated from urine following the oral administration of a test dose of nicotinic acid (3, 4, 6, 7), these derivatives of nicotinic acid should also be determined for the proper evaluation of the urinary nicotinic acid values. On the other hand, the distribution of trigonelline is ubiquitous in nature (10) and, since the compound possesses no anti-blacktongue activity (9), that excreted as a result of direct dietary intake (11) should be excluded from the over-all urinary nicotinic acid values. Quantitative data concerning the natural distribution and urinary excretion of trigonelline are lacking. In the present study it will be shown that by varying the hydrolytic procedures it will be possible to determine which of the nicotinic acid derivatives are excreted. Although the results are still semiquantitative, the procedures used are simple, so that it now becomes possible to carry out extensive studies of the urinary excretion of nicotinic acid and its derivatives (11).

The non-specificity of the urinary values has been found to be due for the most part to the pyridine and nicotine in the specimens reacting with the reagents. Vitamin B_6, like trigonelline, fails to give a positive test.

Yield of "Nicotinic Acid" from Same Urine Sample Subjected to Varying Hydrolytic Procedures—Urine samples with and without added nicotinamide, nicotinuric acid, and trigonelline were subjected to hydrolysis with different concentrations of acid and

alkali for varying periods. A typical series of such tests, taken from a number of similar experiments, is presented in Table II.

TABLE II

Variability in Yield of "Nicotinic Acid" from Same Urine Sample Subjected to Acid and Alkaline Hydrolysis; Recovery of Added Pyridine Compounds

Reagent	Normality	Duration of hydrolysis*	"Nicotinic acid" in sample†	Recovery of added pyridines as nicotinic acid‡		
				Nicotinamide	Nicotinuric acid	Trigonelline
		hrs.	γ	*per cent*	*per cent*	*per cent*
HCl	2.5	0.5	66	99	65	0
	4	0.5	64	102	68	0
	6	0.5	67	98	72	0
	4	5.0	61	98	95	0
NaOH	2.5	0.5	126	96	101	4
	4	0.5	246	98	99	11
	6	0.5	362	97	106	18
	9	0.5	484	102	108	30
	2.5	1.0	196	99	101	8
	4	1.0	310	99	105	17
	6	1.0	376	97	100	20
	9	1.0	490	95	95	30
	2.5	2.0	230	97	105	10
	4	2.0	340	95	94	19
	6	2.0	380	86	90	21
	9	2.0	452	83	85	27
	2.5§	24	378	97	99	19

* The hydrolyses were carried out in graduated test-tubes immersed in a boiling water bath.

† In each case a ½ hour aliquot of a 48 hour urine specimen from a normal subject was subjected to the hydrolysis before and after the addition of the extra pyridine compounds.

‡ To the urine samples subjected to acid hydrolysis the amounts of the added pyridine compounds in each case were equivalent, on the basis of comparative molecular weights, to 200 γ of nicotinic acid; to those subjected to alkali hydrolysis, 1000 γ equivalents were added.

§ In this case the hydrolysis was allowed to proceed under a reflux, so that no change in volume took place. In all the other cases the solutions were simply made up to the indicated normality with no attempt to prevent volatilization during the periods of hydrolysis.

It will be observed that following any of the acid or alkaline hydrolyses[1] of from ½ to 1 hour's duration there is a quantitative conversion of nicotinamide into the free acid, as indicated by the recovery values. However, the initial yields of "nicotinic acid" from the same urine sample subjected to alkaline hydrolysis are far in excess of those obtained by hydrolysis with hydrochloric acid of equivalent normality and increase progressively with increasing concentrations of the alkali.[2] The acid hydrolyses yield relatively constant values despite variations in the concentration of the hydrochloric acid and in the period of hydrolysis. The above results have been confirmed repeatedly (11).

Inasmuch as blank determinations, by the same hydrolytic procedures, have given negative color tests, the increasing yields of "nicotinic acid" with use of increasing concentrations of the alkali cannot be attributed to contaminants in the reagents. Apparently there is normally excreted into the urine a pyridine compound characterized by its resistance to strong acid hydrolysis and its susceptibility to alkaline hydrolysis to yield a compound which then reacts similarly to nicotinic acid with the cyanogen bromide and aniline reagents.

Influence of Various Hydrolytic Procedures upon Subsequent Reactions of Pyridine, Nicotine, and Vitamin B_6 with Reagents—When pure aqueous or urine solutions of pyridine and nicotine are subjected to the acid hydrolyses, constant values for the added pyridine compounds with quantitative recoveries are obtained.

[1] The hydrolyses of the half hour aliquots of the urine specimens were carried out in graduated test-tubes immersed in a boiling water bath. The urine samples subjected to acid hydrolysis in a total volume of 15 cc. were analyzed according to the published procedures (2). Those subjected to alkaline hydrolysis of 2.5 and 4 N were brought to a total volume of 15 cc., while the more alkaline hydrolysates (6 and 9 N) measured 10 cc. To each of the alkaline solutions 10 cc. of ethyl alcohol were added, followed by neutralization with concentrated hydrochloric acid in the cold until the pH was adjusted to 7, and the mixture was then brought to a volume of 30 cc. Exactly 0.1 gm. of the Darco charcoal was added and the suspension filtered. 3 cc. quantities (10 per cent aliquots) of the filtrates were then treated in the same manner as the neutralized filtrates obtained after acid hydrolysis.

[2] The values obtained, although varying widely with differences in the normality of the sodium hydroxide used, are reproducible following each of the alkaline hydrolyses and therefore valid.

When the same solutions are subjected to alkaline hydrolyses, no increase in the "nicotinic acid" values is obtained. In fact, smaller values are generally observed because of volatilization of the added pyridine compounds. Vitamin B_6, when subjected to the same hydrolytic procedures in pure aqueous solution or when added to urine, fails to give a positive color test.

Urinary Excretion of Nicotinuric Acid—Examination of Table II indicates that nicotinuric acid is not the compound responsible for the increased yield of "nicotinic acid" when urine samples are subjected to hydrolysis with increasing concentrations of alkali. The recovery values remain constant and represent complete conversion of the added nicotinuric acid to nicotinic acid as in those tests with the added nicotinamide. However, nicotinuric acid is much more stable to acid hydrolysis than nicotinamide. Prior to hydrolysis the conjugate, in terms of the nicotinic acid moiety, gives 62 per cent of the color produced by free nicotinic acid (see Table I). When subjected to the acid hydrolytic procedures of ½ hour's duration, there is only a slight increase in the yield of free nicotinic acid. Only when the hydrolyses with 4 N hydrochloric acid are allowed to proceed for a period of from 4 to 5 hours are constant values obtained, with quantitative conversions of the conjugate to free nicotinic acid occurring. Repeated recovery experiments with nicotinic acid and nicotinamide added to urine have indicated no volatilization of nicotinic acid during the period of prolonged acid hydrolysis. Normally the values obtained by acid (4 N) hydrolysis of a urine sample for ½ hour are practically the same regardless of whether a short or long period of hydrolysis is employed (11). Increased values following the latter hydrolytic procedure are obtained only when the subject receives an extra test dose of nicotinic acid. In these cases, as in the recovery experiments with added nicotinuric acid, prolonging the period of acid (4 N) hydrolysis gives increasing values, with maximal and constant figures obtained only after the 4 to 5 hour periods of hydrolysis. These findings have been interpreted as indicative of no urinary excretion of nicotinuric acid unless relatively large doses of extra nicotinic acid have been ingested (11).

Urinary Excretion of Trigonelline—We have found much evidence to indicate that the pyridine compound excreted in the urine, which is completely resistant to acid but susceptible to

alkaline hydrolysis, is trigonelline. Increasing both the normality of the acid and the period of acid hydrolysis fails to augment the urinary nicotinic acid value despite the addition of a relatively large amount of trigonelline to the sample (see Table II). With use of increasing concentrations of the alkali, however, there is an increase in the percentage conversion of trigonelline to the nicotinic acid-like reacting substance. This conversion closely parallels the increasing yields of "nicotinic acid" when the same urine samples alone are subjected to the alkaline hydrolyses. Thus, in the series presented, the ratio of the increases in yields of "nicotinic acid," over and above the constant values obtained by acid hydrolysis, following the ½ hour periods of hydrolysis with sodium hydroxide of increasing normality was 1:3:5:7. This ratio was practically the same as that obtained for the percentage recoveries of the added trigonelline expressed as nicotinic acid. Furthermore, extension of the 2.5 and 4 N alkaline hydrolyses of urine over longer periods of time leads to still greater increases in the yield of "nicotinic acid" and this is associated with comparably greater increases in the percentage hydrolysis of the added trigonelline. In the more alkaline hydrolysates these increases in the initial yield of nicotinic acid or in the percentage conversion of the added betaine to the nicotinic acid-like reacting substance are not apparent. Apparently under such conditions the increased hydrolysis of the trigonelline is masked by concomitant destruction of the end-product.

The pyridine compound in the urine, resistant to acid but susceptible to alkaline hydrolysis, also behaves like trigonelline with charcoal and zeolite adsorbents. The addition of charcoal[3] to either a neutral urine sample or to a pure aqueous solution of trigonelline is followed in each case by almost quantitative adsorption of the compound; this was indicated by no appreciable increase in the yields of nicotinic acid as a result of subsequent alkaline hydrolysis. Passage of either a pure aqueous solution of trigonelline or of the half hour urine sample through a permutit[4] column (12) results in no appreciable adsorption of the trigonelline;

[3] Darco, a vegetable charcoal, obtained from the Coleman and Bell Company, Norwood, Ohio.

[4] Delcalso, approximately 50 mesh, kindly furnished by the Permutit Company, New York.

the filtrates gave values following alkaline hydrolysis which were the same as those recorded initially for the solutions.

The betaine *in pure aqueous solution* is resistant to all of the alkaline hydrolyses. Accordingly, in the adsorption experiments with pure solutions of trigonelline it was essential to add to the filtrates ½ hour urine samples and compare the results with those obtained when no adsorptions were involved. The concentration of the urinary solids influences the extent of hydrolysis. Tests, in which one-fifth as much urine was used but in which total volume, normality, and duration of the alkaline hydrolysis were the same, gave only one-seventh of the previous values. A comparable decrease in the percentage hydrolysis of the trigonelline added to the smaller urine aliquots was also noted. Just what in urine makes possible the alkaline hydrolysis of the betaine has not been determined. The addition of the pyridine compounds listed in Table I to pure aqueous solutions of trigonelline failed to catalyze the conversion of the betaine to the nicotinic acid-like reacting substance.

Procedures Used in Chemical Study of Urinary Excretion of Nicotinic Acid and Its Derivatives—The 24 hour urine sample is collected in a bottle containing 30 cc. of 3.5 N sulfuric acid. Three ½ hour aliquots are concentrated over a steam bath, while at the acid reaction, to a suitable volume. These are then subjected to to different hydrolytic procedures: (*a*) one with 4 N hydrochloric acid for a period of ½ hour, (*b*) the second with the same concentration of the acid for a period of 5 hours, and (*c*) the third with 9 N sodium hydroxide for a period of ½ hour.[5]

The value for the sample subjected to the short period of acid hydrolysis is believed to include all the voided nicotinic acid, nicotinamide, free pyridine, nicotine, two-thirds of the nicotinuric acid,[6] and any other unknown pyridine compounds which react

[5] The samples subjected to the acid hydrolyses are brought to a total volume of 15 cc., but no attempt was made to maintain a constant volume during the period of hydrolysis. Those subjected to alkaline hydrolysis are made up to a total volume of 10 cc. The hydrolysates are then treated as described in foot-note 1 of this paper.

[6] This is due to the fact that nicotinuric acid itself, although not appreciably hydrolyzed during the short period of acid hydrolysis, reacts directly with the reagents to give a color the intensity of which is equal to 62 per cent of that obtained with an equivalent amount of nicotinic acid.

with the reagents. The values obtained after the prolonged acid hydrolysis include, in addition to the above, all of the nicotinuric acid because of complete hydrolysis. Accordingly, from the increment obtained from the longer hydrolysis an estimation can be made of the total nicotinuric acid present.

The value obtained after alkaline hydrolysis of the urine sample is used for the estimation of trigonelline in urine. This figure includes also the pyridine compounds determined by use of the prolonged acid hydrolysis. The results following alkaline hydrolysis can then be corrected to yield values due solely to the trigonelline. More than twenty recovery experiments have been carried out with the betaine added to urine samples from six individuals. The percentage conversion of the added trigonelline to the nicotinic acid-like reacting substance varied from 28 to 38 per cent and the values in each case were reproducible; the average conversion factor was 33 per cent. We have, therefore, interpreted the increased yield of nicotinic acid, over and above that following the 5 hour period of acid hydrolysis, as representing one-third of the excreted trigonelline.

Because of the large conversion factors involved in estimating the total amounts of the nicotinic acid derivatives excreted, the results should be considered only as semiquantitative. However, the procedures appear to be specific for these compounds and because of their simplicity lend themselves to routine examination of urine samples (11). In addition, the results are reproducible. For publication purposes, only the values actually obtained by the direct chemical analyses are given. These are sufficient in themselves to indicate which of the nicotinic acid derivatives are excreted and their relative quantities. From the data presented approximations can be made of the absolute amounts of these compounds present in a given sample.

Trigonelline As a Normal Constituent of Urine; Effect of Coffee and Smoking upon Urinary "Nicotinic Acid" Values—There is a wide-spread distribution of trigonelline and pyridine-containing alkaloids in the plant kingdom (10); the common beverages (coffee, tea, and cocoa) are considered to be relatively rich in some of these substances. There is also a suggestion in the literature (6) that confirmed smokers may develop the ability to convert

nicotine to trigonelline as a detoxicating measure. The excretion of nicotine, as the unchanged alkaloid, in the urine of smokers has been reported (5).

TABLE III

Effect of Coffee Drinking and Smoking upon Urinary "Nicotinic Acid" Values

The results are given in mg. per 24 hours.

Subject	Experiment	Urinary "nicotinic acid" values after hydrolysis		
		½ hr., 4 N HCl	5 hrs., 4 N HCl	½ hr., 9 N NaOH
D. M.	Basal diet + no coffee or smoking 3 days prior to and during 24 hrs. of urine collection	3.7	3.3	11
	Basal diet + 6 cups black coffee (no smoking)	3.6	3.4	70
W. R.	Basal diet + no coffee or smoking 3 days prior to and during 24 hrs. of urine collection	4.2	3.6	13
	Basal diet + 6 cups black coffee (no smoking)	4.5	4.2	91
	After 4 days adjustment period, basal diet + smoking, 30 cigarettes (no coffee drinking)	7.8	7.6	58
N. A.	Normal routine; basal diet + smoking, 20 cigarettes (no coffee drinking)	15.1	14.9	34
	24 hr. period following above, smoking discontinued (no coffee drinking)	10.8	9.5	23
	Subsequent 24 hr. period, smoking still discontinued (no coffee drinking)	4.7	4.3	17
	First 24 hr. period after return to normal routine (no coffee drinking)	9.8	9.3	29

Studies were accordingly carried out to ascertain the influence of smoking and coffee consumption upon the urinary "nicotinic acid" values. Representative data are presented in Table III.

The constant excretion values obtained with the two subjects subsisting on the basal diets alone are given. These figures were

reproducible not only for the same urine samples but also for those collected on consecutive days while the individuals continued on the same dietary régime. The larger values consistently obtained with the samples subjected to alkaline hydrolysis indicate trigonelline to be a normal constituent of urine. This conclusion is supported by the isolation of the betaine from normal human urine despite the complete abstinence from smoking and from coffee, tea, and cocoa consumption (6). The figures for the 24 hour period associated with coffee drinking showed no change with acid hydrolysis but a tremendous increase following alkaline hydrolysis. This increase is attributed to the extra excretion of trigonelline.

Studies of the urinary excretion of nicotinic acid and its derivatives have been carried out with eleven normal subjects who pursued a normal but constant daily routine (11). Of the six individuals in this group, who smoked regularly, three had urinary "nicotinic acid" values following acid hydrolysis not much greater than those frequently obtained in similar tests of urines from nonsmokers. The other three smokers consistently gave very much higher values. Representative data of studies conducted with subjects from each of these two groups of smokers are also presented in Table III. With subject W. R., the major increase in the urinary "nicotinic acid" values after smoking was observed in the sample subjected to alkaline hydrolysis. In the case of the other individual, N. A., the values which responded to changes in smoking habits were those obtained following acid hydrolysis of the samples with only parallel changes in the values after alkaline hydrolysis. Apparently subjects differ in the way they handle nicotine, some converting it to trigonelline, others excreting it primarily as the unchanged alkaloid[7] (which is quantitatively determined along with the other pyridines).

[7] Additional tests have indicated that free nicotine is excreted by such individuals. If, in the preliminary concentration of the ½ hour urine sample, the acid reaction is first neutralized to pH 7, most of the free pyridine is volatilized; the nicotine, however, is not affected. Such tests carried out with urine samples from smokers in this group gave values not appreciably less than those previously obtained when the samples were concentrated at the usual acid reaction.

SUMMARY

Nicotinamide is stable in acidified urine for a period of more than 30 days. The urinary pyridine compounds vary widely in the extent of their reactions with the cyanogen bromide and aniline reagents. Procedures are described for the quantitative conversion of nicotinamide and nicotinuric acid to nicotinic acid. Of the two, nicotinuric acid is more stable to acid hydrolysis. The excretion of this compound can be estimated by making use of the increment in the urinary nicotinic acid value following prolonged acid hydrolysis. There is normally excreted a pyridine compound, characterized by its complete resistance to acid hydrolysis but susceptibility to alkaline hydrolysis, yielding a substance which reacts subsequently like nicotinic acid with the reagents. The evidence suggesting that this is trigonelline is the parallel behavior of the compounds when exposed to acid and alkaline hydrolyses of varying normality and duration, similar adsorption properties, and dependency upon some urinary constituent for catalysis in the alkaline hydrolysis of the compounds. The extent of trigonelline excretion may be estimated from the increase in the urinary "nicotinic acid" value after alkaline hydrolysis, over and above that obtained following prolonged acid hydrolysis. Coffee consumption leads to a markedly increased excretion of trigonelline with no change observed in the urinary nicotinic acid values obtained during acid hydrolysis. Smoking in the case of one group of individuals augments mainly the urinary excretion of trigonelline, while in the other free nicotine is excreted and this value is included in the total urinary "nicotinic acid" figure. Vitamin B_6, before and after exposure to all the hydrolytic procedures described, fails to give a positive color test with the reagents.

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URINARY EXCRETION OF NICOTINIC ACID AND ITS DERIVATIVES BY NORMAL INDIVIDUALS*

By DANIEL MELNICK,† WILLIAM D. ROBINSON,‡ AND HENRY FIELD, Jr.

(From the Department of Internal Medicine, Medical School, University of Michigan, Ann Arbor)

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In the preceding paper (1) a study was made of the pyridine compounds found in urine in order to determine to what extent their excretion influences the chemically determined (2) urinary "nicotinic acid" values. Procedures were described for the estimation of nicotinuric acid and trigonelline in urine based upon the relative stabilities of these compounds to acid and alkaline hydrolysis. After prolonged acid hydrolysis nicotinuric acid is quantitatively converted to nicotinic acid and only after strong alkaline hydrolysis is trigonelline converted to a nicotinic acid-like reacting substance. The values obtained are reproducible and the procedures appear to be specific for these nicotinic acid derivatives. However, because of the admittedly semiquantitative character of the calculated figures from use of large conversion factors, only the total values obtained by direct chemical analyses are given in this paper.

Some pyridine compounds other than nicotinic acid and the above derivatives are included in the *total* urinary "nicotinic acid" values (1). Excretion of these pyridine compounds may mask differences between normal and deficient individuals with respect to the true nicotinic acid values of their urines. The evaluation of the *extra* excretion of nicotinic acid and its derivatives, after the

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† Upjohn Fellow in Clinical Research, 1937–41.

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oral administration of a test dose to subjects following a constant daily routine, offers a means for correcting for the initial lack of specificity of the chemical method. The present report deals with the range of values obtained in such studies conducted with normal individuals. Data are presented showing in what forms nicotinic acid is excreted in the urine, the prompt conversion of most of the nicotinic acid to these derivatives, and rapidity of excretion of the pyridine compounds after dosage. Differences in metabolic behavior of nicotinic acid and nicotinamide are also indicated.

EXPERIMENTAL

Response of Normal Individual to Oral Test Dose of Extra Nicotinic acid—Eleven adult subjects of varying size were used for this study. Selection of foods during the test period was left to the choice of the subject and represented what that individual generally ate. Smoking and coffee consumption were not restricted but were maintained constant during the test period.[1] Three consecutive 24 hour urine samples were collected.[2] Following the 24 hour basal period and just prior to the collection of the second sample, an aqueous solution of 500 mg. of nicotinic acid was taken orally at the completion of the largest meal of the day. The two subsequent 24 hour urine samples were collected. The results of the analyses of the three consecutive urine samples are given in Table I. Each sample was subject to three types of hydrolysis, as indicated. The increase in the nicotinic acid value after the prolonged period of acid hydrolysis is due to hydrolysis of nicotinuric acid and the increment after alkaline hydrolysis comes from the trigonelline fraction (1). The value for the *extra* urinary "nicotinic acid" excretion following the administration of the test dose was calculated by subtracting from the total figure, obtained for the first 24 hour period after dosage, the average of the values for the immediately preceding and following 24 hour periods.

Examination of the data in Table I shows that there is normally no excretion of nicotinuric acid in urine; this is indicated by no increase in the values obtained for the basal 24 hour urine samples

[1] This was necessary because of the influence of these factors upon the urinary "nicotinic acid" values (1).

[2] The urine collection periods began in each case after dinner.

TABLE I

*Urinary Excretion (in Mg.) of Nicotinic Acid and Its Derivatives by Normal Individuals before and after Oral Administration of 500 Mg. of Nicotinic Acid**

Subject	Sex	Weight	Height	Surface area	Urinary "nicotinic acid" values after hydrolysis†											
					Prior to oral dose			After oral dose						Excretion of oral dose‡		
					½ hr., 4 N HCl	5 hrs., 4 N HCl	½ hr., 9 N NaOH	½ hr., 4 N HCl	5 hrs., 4 N HCl	½ hr., 9 N NaOH	½ hr., 4 N HCl	5 hrs., 4 N HCl	½ hr., 9 N NaOH	½ hr., 4 N HCl	5 hrs., 4 N HCl	½ hr., 9 N NaOH
					Per 24 hrs.			Per 1st 24 hrs.			Per 2nd 24 hrs.			Per 1st 24 hrs.		
		kg.	*cm.*	*sq.m.*												
E. W.	F.	53.5	161	1.55	2.3	2.3	18	57.6	68.7	110	3.3	3.4	31	54.8	65.8	85
O. B.§	M.	54	161	1.56	12.9	12.9	33	71.7	85.0	128	15.0	13.5	26	57.7	71.8	98
M. J.	F.	56	162	1.59	2.0	1.7	12	86.0	110.0	132	2.7	2.5	9	83.6	107.9	121
C. M.	M.	68	178	1.84	2.1	1.8	12	38.4	52.3	72	2.9	2.4	17	35.9	50.2	57
N. A.§	"	71	174	1.85	16.4	16.5	29	39.3	49.7	81	18.4	18.4	26	21.9	32.2	53
A. M.	F.	72	169	1.82	2.0	1.9	3	48.0	62.4	79	2.4	2.4	4	45.8	60.2	75
W. R.§	M.	72.5	178	1.90	7.1	6.5	43	32.4	41.2	96	8.6	8.0	44	24.5	33.9	52
D. M.	"	74	170	1.85	3.1	3.0	28	28.0	38.6	69	4.0	3.6	27	24.4	35.3	41
S. S.§	"	75	178	1.91	5.1	4.4	30	40.8	50.6	107	6.0	5.9	32	35.2	45.4	76
H. H.§	"	85	188	2.12	6.7	6.3	32	26.9	35.7	74	6.5	5.5	30	20.3	29.8	43
R. K.§	"	86	187	2.12	29.3	28.1	71	75.7	92.6	178	30.7	28.6	75	45.7	64.2	105

* The test dose of nicotinic acid in aqueous solution was taken immediately after the largest meal of the day. The three consecutive 24 hour periods, during which the urine samples were collected, began in each case after dinner.

† The significance of the different types of hydrolysis, indicating which of the nicotinic acid derivatives is excreted, is explained in the text.

‡ These figures were calculated by subtracting from the total values, obtained for the first 24 hour period subsequent to the administration of the test dose, the averages of the values for the immediately preceding and following 24 hours.

§ These individuals are habitual smokers, smoking from twenty to thirty cigarettes daily.

when subjected to prolonged acid hydrolysis. Trigonelline is a normal constituent of urine; in each case a definitely larger value for the urinary excretion of nicotinic acid is obtained following the period of alkaline hydrolysis of the sample.[3] The presence of trigonelline in urine is due to both passive excretion of the betaine following its consumption as such in the diet[4] and that arising, as a detoxication measure, when large doses of nicotine (1, 3), nicotinic acid, and possibly other pyridine compounds are taken. The average increased urinary "nicotinic acid" value following alkaline hydrolysis of the basal urine samples in the series presented is 20 mg. per 24 hours. This represents a daily excretion of approximately 60 mg. of trigonelline (1). Values for the basal 24 hour urinary "nicotinic acid" values following acid hydrolysis vary widely, from 1.7 to 29.3 mg. The three samples yielding very large values were from smokers who excreted nicotine principally as the unchanged alkaloid (1, 4). Studies in which volatilization of the pyridine compounds by steam distillation of the urine samples at various pH values was employed have indicated that free pyridine is also present in some to an appreciable extent. There is a definite tendency for the values obtained during the basal periods to be less following prolonged acid hydrolysis compared with those obtained after the short period of acid hydrolysis. Repeated recovery experiments have shown that the decrease is due to volatilization or destruction of a urinary pyridine compound other than those studied ((1) Table I). It is thus apparent that the "nicotinic acid" values for the basal periods include pyridine compounds other than nicotinic acid. The true urinary nicotinic acid is believed to be in the neighborhood of the lower normal range.

[3] Only subject A. M., one of the non-smokers and non-coffee consumers, failed to show the very large increment in the urinary "nicotinic acid" value following alkaline hydrolysis. The small increment in this case, however, is real; recovery experiments with trigonelline added to ½ hour aliquots of the same urine sample gave the usual 33 per cent conversion of the betaine to the nicotinic acid-like reacting substance.

[4] When one of the standardized subjects (D. M., Table II) took a postprandial oral test dose of trigonelline, equivalent to 350 mg. of nicotinic acid, the increase in the urinary nicotinic acid value occurred only in the sample subjected to alkaline hydrolysis. The increase represented an extra excretion of 135 mg. of trigonelline, all within the first 24 hours after dosage. (The trigonelline was furnished by General Biochemicals, Inc.)

The oral administration of the test dose of extra nicotinic acid is followed by a rapid and marked increase in the urinary "nicotinic acid" value. Within 24 hours after dosage the values for nicotinic acid and its derivatives in the urine are back to normal; the urinary values during the following 24 hour period are practically the same as the basal figures. In each case there is a marked urinary excretion of nicotinuric acid after the oral administration of the test dose (increase in values following prolonged acid hydrolysis). It has been shown (1) that this increment in the urinary "nicotinic acid" values represents only one-third of the nicotinuric acid present.[5] Thus, it is readily noted that after dosage most of the extra urinary nicotinic acid excretion, determined after prolonged acid hydrolysis of the samples, is due to the presence of nicotinuric acid. Some individuals, such as N. A. and H. H., excreted practically all the nicotinic acid, so determined, as the glycine conjugate. When the urinary "nicotinic acid" values are very large, due to the intake of a relatively large dose of the compound by the smaller individuals, such as E. W. and O. B., or when the test dose is taken by the fasting subject (see Fig. 2), appreciable amounts of free nicotinic acid (or amide) are excreted. In each case, listed in Table I, there is a marked but variable excretion of extra trigonelline (increase in values following alkaline hydrolysis) after dosage. This increment should be multiplied by 3 for the estimation of the absolute excretion of the betaine (1).

The values listed for the extra excretion of nicotinic acid and its derivatives after the administration of the test dose show a tendency of the smaller individuals to excrete more of the pyridine compounds. However, individual variation in the handling of the test dose makes this correlation poor. The average values obtained in this study are 40.9 mg. of extra "nicotinic acid" following the short period of acid hydrolysis, 54.2 mg. after prolonged acid hydrolysis, and 73 mg. following alkaline hydrolysis. Calculations, based upon the fundamental concepts set forth in the preceding paper (1), indicate a total extra excretion of 110 mg.

[5] This is due to the fact that nicotinuric acid, although not appreciably hydrolyzed during the short period of acid hydrolysis, reacts directly with the reagents to give a color the intensity of which is equal to 62 per cent of that obtained with an equivalent amount of nicotinic acid. The value obtained after prolonged acid hydrolysis, however, does include all the voided nicotinuric acid expressed as free nicotinic acid.

of nicotinic acid and derivatives, or 22 per cent of the test dose. 51 per cent of the increased urinary excretion of nicotinic acid is in the form of trigonelline, 36 per cent is in the form of nicotinuric acid, and only 13 per cent in the form of free nicotinic acid or amide.

Reproducibility of Values for 24 Hour Urinary Excretion of Nicotinic Acid and Derivatives before and after Administration of

TABLE II

Reproducibility of Basal Values for 24 Hour Urinary Excretion of Nicotinic Acid and Its Derivatives*

The results are expressed in mg. per 24 hours.

Subject	No. of samples analyzed†	Values obtained after hydrolysis‡								
		½ hr., 4 N HCl			5 hrs., 4 N HCl			½ hr., 9 N NaOH		
		Range	Average	Standard deviation	Range	Average	Standard deviation	Range	Average	Standard deviation
C. M.	4	2.1- 3.4	2.9	0.6	1.8- 2.9	2.4	0.5	12-19	17	3.3
E. W.	16	1.6- 3.9	2.8	0.6	1.5- 3.6	2.5	0.6	18-32	24	4.8
D. M.	18	2.4- 5.5	3.6	0.7	2.2- 5.2	3.2	0.6	18-30	26	3.4
W. R.§	10	5.3- 8.6	7.0	0.9	4.0- 8.0	6.6	1.1	27-64	43	12.2
H. F.§	5	24.3-30.2	27.4	2.6	23.3-31.7	27.3	3.5	53-80	67	10.8

* These were obtained when the diet furnished the sole intake of nicotinic acid and its derivatives.

† These samples were collected over a period of 6 months and were spaced at least 1 week apart.

‡ The significance of the different types of hydrolysis, indicating which of the nicotinic acid derivatives is excreted, is explained in the text.

§ These individuals are habitual smokers, smoking from twenty to thirty cigarettes daily.

Test Dose of Extra Nicotinic Acid—In Table II are summarized the results of the analyses of 53 basal 24 hour urine samples from five normal individuals. Each sample was subjected to the three types of hydrolysis, as indicated. The values, though extending over an appreciable range, are within definite limits characteristic for each of the subjects. Much of the variation noted is undoubtedly due to no attempt having been made by these individuals to follow a constant dietary régime during the urinary collection

periods. When the diet and daily routine are maintained constant, the urinary "nicotinic acid" values are reproducible (1). The average basal excretion values listed in Table II have been used to correct the total figures obtained when the same individuals received test doses of nicotinic acid and nicotinamide. The results have been plotted (Figs. 1 to 3) as extra urinary "nicotinic acid" excreted.

In Fig. 2 are presented the results for repeated determinations of the extra urinary excretion of nicotinic acid and its derivatives when the normal subject takes the 500 mg. test dose of nicotinic acid after eating. The average deviation of the two values in a set from its average figure is ± 9 per cent, with a maximal deviation of ± 18 per cent. These results indicate that the values obtained are sufficiently reproducible to make the data in Table I valid and allow significance to be attached to the differences in the urinary excretion of nicotinic acid when varying test doses are taken (Fig. 1), when the test dose is taken by the subject fasting rather than after eating, and when nicotinamide is taken in place of the free acid (Figs. 2 and 3).

Correlation between Urinary "Nicotinic Acid" Values and Size of Test Dose—The first 24 hour urine samples were collected from two normal subjects after they had received varying oral test doses of nicotinic acid after the largest meal of the day.[6] The extra urinary excretions of nicotinic acid and derivatives are indicated in Fig. 1. Only small amounts of these pyridine compounds are excreted when the dose of extra nicotinic acid is 3.4 mg. per kilo of body weight. In these tests the extra urinary "nicotinic acid" values (with calculations made to convert the results obtained after hydrolysis into absolute quantities of the nicotinic acid derivatives) account for only 3 and 5 per cent of the test doses. With larger test doses there is not only an absolute increase in these urinary pyridine compounds but also a marked augmentation in the percentage of the test doses excreted. Thus, when the dose of extra nicotinic acid was 9.4 mg. per kilo of body weight, the urine values accounted for 25 and 14 per cent of the test dose.

Urinary Excretion of Nicotinic Acid and Its Derivatives When Test Dose Is Taken by Fasting Subject. Difference in Urinary

[6] Approximately 10 days were allowed to lapse between each of the periods when the test doses were taken.

Excretion Values When Nicotinamide Is Taken—Test doses of nicotinic acid were administered orally to three of the normal subjects, but this time the solutions were taken 12 hours after dinner and 3 hours prior to breakfast. The results are plotted in Fig. 2. For comparative purposes the values obtained when these same subjects received the same test doses after eating are

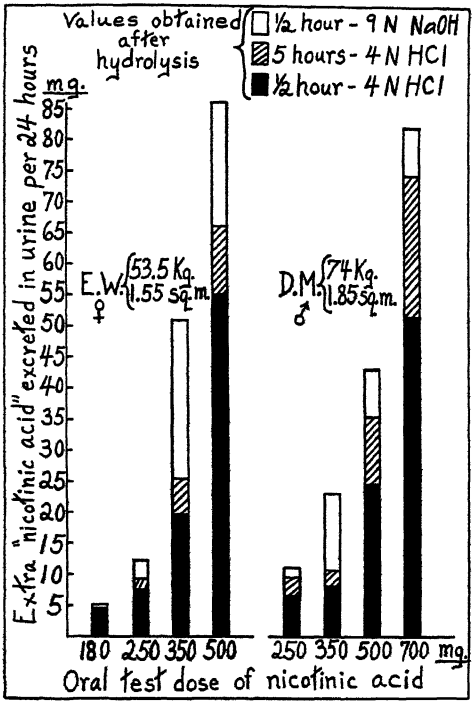

Fig. 1. Correlation between urinary excretion and size of the test dose of extra nicotinic acid. The plotted values have been corrected for the basal excretions before the test doses were administered. The significance of the different types of hydrolysis is explained in the text.

also presented. It will be observed that there is a marked increase in the extra urinary "nicotinic acid" values (from 100 to 300 per cent, when calculations are made to convert the values obtained after hydrolysis into absolute quantities of the nicotinic acid derivatives). The "flooding" effects, resulting from taking the test dose while fasting, are also apparent from the values

calculated to yield the per cent of total pyridines excreted as free nicotinic acid (or amide); 40 per cent in the present series compared with only 13 per cent when the test dose is taken after dinner.

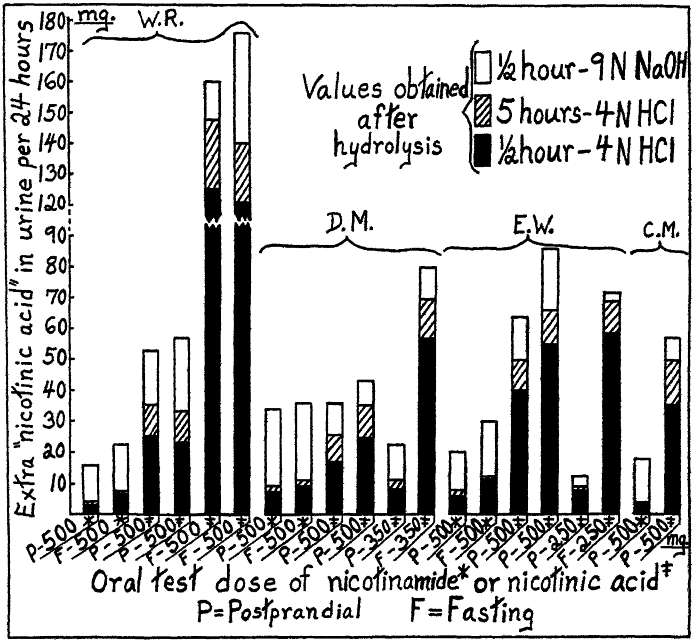

FIG. 2. Difference in the values for the extra urinary excretion of nicotinic acid and its derivatives when the test dose of nicotinic acid or amide is taken immediately after a meal or while fasting. The plotted values have been corrected for the basal excretions before the test doses were administered. The significance of the different types of hydrolysis is explained in the text.

When nicotinamide is taken by the same subjects under the same experimental conditions, the extra urinary "nicotinic acid" values are generally very much less[7] than those obtained when

[7] There is one exception in the series presented. In the case of D. M., the extra urinary "nicotinic acid" value, when calculations were made to convert the results obtained after hydrolysis into absolute quantities of the nicotinic acid derivatives, was appreciably greater following the use of nicotinamide as the postprandial test dose. This was due to the relatively much greater excretion of trigonelline.

nicotinic acid constitutes the test dose (Fig. 2). From 80 to 90 per cent of the extra nicotinic acid in the urine is calculated to be present as trigonelline. No appreciable difference in the urine values is obtained when the test dose of nicotinamide is taken by the subject after eating or while fasting.

Rapidity of Conversion of Nicotinic Acid into Nicotinuric Acid and Trigonelline Following Its Oral Administration, and Prompt Excretion of These Compounds—Fractional urine samples were

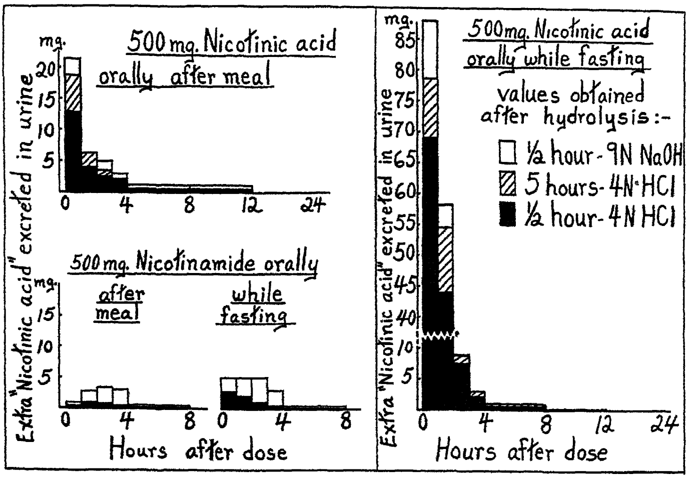

FIG. 3. Partition of the 24 hour extra urinary excretion of nicotinic acid and its derivatives by the same normal subject receiving 500 mg. of nicotinic acid or its amide orally immediately after a meal or while fasting. The plotted values have been corrected for the basal excretions before the test doses were administered. The significance of the different types of hydrolysis is explained in the text.

collected during the first 24 hours after the oral administration of test doses of nicotinic acid or nicotinamide to the normal subject, W. R. (Fig. 2), and analyzed separately. The results are presented in Fig. 3. When the 500 mg. sample of nicotinic acid was taken orally by the subject, either before eating or while fasting, there was a prompt increase in the urinary "nicotinic acid" values. Practically all the excreted pyridines are voided within 4 hours after administration of the test dose; the maximal excretion is within the 1st hour. When the same quantity of nicotinamide is

taken as the test dose, a more gradual and much smaller excretion of nicotinic acid and its derivatives is observed. In all cases there occurs a prompt and continuous conversion of nicotinic acid to nicotinuric acid and trigonelline. The blood values associated with these urinary "nicotinic acid" figures are presented in Fig. 1 of the following paper (5). The only curve of urinary excretion which fails to parallel the blood values is that obtained following the administration of the oral test dose after eating. The reason for this is discussed elsewhere (5).

SUMMARY

Trigonelline is a normal constituent of urine; nicotinuric acid is not. The basal 24 hour urinary "nicotinic acid" values obtained in a study with eleven well nourished individuals varied from 1.7 to 29.3 mg. Because of the demonstrated non-specificity of the method the true nicotinic acid values are believed to be in the neighborhood of the lower normal range. In addition, the average excretion of nicotinic acid as trigonelline amounts to approximately 60 mg. per 24 hours. The oral postprandial administration of the test dose of 500 mg. of extra nicotinic acid is followed by a rapid and marked increase in the urinary "nicotinic acid" values, almost all of the excreted pyridine being voided within the first 4 hours. The maximal excretion occurs during the 1st hour after the test dose is taken. On the average 110 mg. (22 per cent) of the test dose of nicotinic acid are excreted, 51 per cent in the form of trigonelline, 36 per cent as nicotinuric acid, and 13 per cent as free nicotinic acid or amide. The percentage excretion of the test dose varies directly with the size of the dose. There is an increase of from 100 to 300 per cent in the extra urinary excretion of nicotinic acid and derivatives over that following postprandial doses when the nicotinic acid is taken while fasting. In either case there is a prompt and continuous conversion of nicotinic acid to nicotinuric acid and trigonelline.

When nicotinamide constitutes the test dose, the extra urinary "nicotinic acid" values are generally very much less and indicate no appreciable difference arising from the relation of dosage to the meal. Also, the rate of excretion of the nicotinic acid and derivatives is much more gradual, with trigonelline accounting for from 80 to 90 per cent of the excreted pyridine. The values before

and after the administration of the test doses are sufficiently reproducible to justify the conclusions drawn.

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FACTORS AFFECTING THE CONCENTRATION AND DISTRIBUTION OF NICOTINIC ACID IN THE BLOOD*

By DANIEL MELNICK,† WILLIAM D. ROBINSON,‡ and HENRY FIELD, Jr.

(*From the Department of Internal Medicine, Medical School, University of Ann Arbor*)

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A simple chemical method for the determination of nicotinic acid (or nicotinamide) in blood has been reported (1). The present report deals with the stability of nicotinamide in blood, specificity of the procedures, the range of values for normal adults, and the distribution of the compound between the plasma and corpuscular elements. Typical blood nicotinic acid tolerance curves, following the administration of the compound (or nicotinamide) through various routes, are presented. Such studies with normal and pellagrous individuals may lead to the development of a procedure for the laboratory diagnosis of nicotinic acid deficiency.

EXPERIMENTAL

Stability of Nicotinamide in Blood—To each of the 10 cc. of oxalated blood samples in three separate series 100 γ of nicotinamide were added. In the first series sterile blood was used, the amide being added aseptically; in the second group no aseptic precautions, other than the use of chemically clean apparatus, were employed; and in the last series the samples with and without the added amide were pipetted into the concentrated hydrochloric acid, as described in preparing the samples for hydrolysis (1). The samples stored at 5–8°, were tested daily for nicotinic acid

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† Upjohn Fellow in Clinical Research, 1937–41.

‡ Upjohn Fellow in Clinical Research, 1938–40.

according to the published procedure (1). In all cases the values remained constant over a period of 5 days[1] with the theoretical recoveries of the added nicotinamide obtained.

Effect of Recently Ingested Meal, Coffee, and Smoking upon Blood Nicotinic Acid Level—Inasmuch as these factors have been shown to influence the excretion of nicotinic acid, its derivatives, or the pyridine compounds, which are included in the urinary "nicotinic acid" values (2), tests were carried out to determine

TABLE I

Negligible Effect of Recently Ingested Meal, Coffee, and Smoking upon Blood Nicotinic Acid Level

Subject	Experiment	Blood nicotinic acid
		mg. per cent
D. M.	14 hrs. fasting	0.58
	4 " after dinner, 10 hrs. after lunch	0.59
	14 " fasting	0.64
	1 hr. after drinking 4 cups of coffee (no eating or smoking)	0.68
W. R.	14 hrs. fasting	0.59
	4 " after dinner, 10 hrs. after lunch	0.62
	14 " fasting	0.62
	1 hr. after drinking 4 cups of coffee (no eating or smoking)	0.69
	14 hrs. fasting	0.64
	Immediately after smoking 10 cigarettes consecutively (no eating or coffee drinking)	0.59
D. L.	14 hrs. fasting	0.72
	Immediately after smoking 10 cigarettes consecutively (no eating or coffee drinking)	0.73

their influence upon the blood values. The results, presented in Table I, indicate that the blood nicotinic acid values are unaffected by any of the experimental procedures. However, the tests with alkaline (9 N) hydrolysis of the blood samples for the estimation of trigonelline which might be present were inconclusive. In such analyses turbidities, that could not be corrected for, developed in the final solutions. Also, similar tests with trigonelline added

[1] Tests for the stability of nicotinamide in blood, stored for more than 5 days, were not conducted.

to the samples failed to show any appreciable conversion of the betaine to the nicotinic acid-like reacting substance. Thus, trigonelline may have been present but this method for its estimation is not applicable to blood.

Blood Nicotinic Acid Values of Normal Adults—Blood samples were collected from twenty-five males and thirteen females in the postabsorptive state. The results of the analyses are presented in Table II. There was no correlation between the values and the subjects' dietary and smoking habits. The slightly higher concentrations of nicotinic acid in the blood of men may be due to comparably greater hematocrit values; approximately 90 per cent of the total nicotinic acid is in the corpuscular elements of the blood (Tables III and IV). The values listed in Table II are considerably greater than those obtained by other chemical procedures

TABLE II

Concentration of Nicotinic Acid in Blood of Normal Human Adults

No. of subjects	Sex	Range	Average	Standard deviation
		mg. per cent	*mg. per cent*	*mg. per cent*
25	M.	0.54–0.83	0.69	±0.07
13	F.	0.52–0.74	0.62	±0.06

(3–5) but in good agreement with the biological method of Lwoff and associates (6, 7). Our procedures of direct acid hydrolysis of the blood followed by preferential charcoal adsorption of the pigments eliminates loss of nicotinic acid due either to inadequate methods of extraction (4), improper charcoal decolorization (3, 5), or to use of protein precipitants (3, 5) in the preparation of *filtrates* suitable for hydrolysis. Protein precipitants have been shown to be effective agents in the *precipitation* of the nicotinamide-containing coenzymes of the blood (8, 9). The smaller values obtained by Axelrod and Elvehjem (10) may be due to the use of a yeast fermentation method which is specific for coenzyme I alone and not for total nicotinic acid.

The values for the blood nicotinic acid concentration of the same sample are reproducible to within ±3 per cent of the average. However, the figures obtained at different periods with the same individual following a normal daily routine may vary widely

(*e.g.*, 0.59 to 0.76 mg. per cent) but remain always in the normal range. Theoretical recoveries of added nicotinamide as nicotinic acid are consistently obtained. Tests with pure coenzyme I[2] added to blood also give quantitative recoveries of the pyridine moiety as nicotinic acid. The plasma values, which approximate the lower limit of sensitivity of the method, are reproducible only to within ±15 per cent of the average.

TABLE III

Concentration of Nicotinic Acid in Normal Blood and Plasma and Its Distribution between Plasma and Corpuscles

Subject	Hematocrit	Nicotinic acid			Per cent blood nicotinic acid in corpuscles‡
		Blood*	Plasma*	Corpuscles†	
	per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*per cent*
W. R.	43.0	0.68	0.21	1.30	82
E. W.	42.3	0.67	0.10	1.44	91
H. W.	44.4	0.67	0.13	1.35	90
G. S.	43.0	0.74	0.13	1.56	90
N. A.	43.1	0.79	0.20	1.58	86
D. M.	43.6	0.76	0.11	1.60	92
D. L.	42.2	0.79	0.22	1.56	84
H. H.	43.3	0.79	0.24	1.50	82
C. W.	43.7	0.69	0.05	1.51	96
Average..	43.2	0.73	0.15	1.49	89

* Obtained by direct chemical analyses.

† Calculated from

$$\frac{\text{Blood value} - (\text{plasma value} \times (1 - \text{hematocrit}))}{\text{Hematocrit}}$$

with the hematocrit expressed as a fraction.

‡ These were calculated from

$$\frac{\text{Corpuscle value} \times \text{hematocrit}}{\text{Blood value}}$$

Distribution of Nicotinic Acid in Blood—In Table III are presented the values obtained with nine normal subjects for the concentration of nicotinic acid in the blood and plasma of the same sample. Calculations indicate that approximately 90 per

[2] The authors are indebted to Dr. C. A. Elvehjem and Dr. M. A. Lipton of the University of Wisconsin for furnishing them with the pure coenzyme I preparation.

cent of the blood nicotinic acid is in the corpuscles. This confirms the observations made by Kohn and Bernheim (11) and Dorfman and associates (12) using bacterial growth methods.

Inasmuch as most of the blood nicotinic acid is in the cells, the influence of pathological variations in the hematocrit upon the

TABLE IV

Concentration of Nicotinic Acid in Blood, Plasma, and Corpuscles of Subjects with Abnormal Hematocrit Values

Subject	Status	Hematocrit	Nicotinic acid			Per cent blood nicotinic in acid corpuscles*
			Blood*	Plasma*	Corpuscles*	
		per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*per cent*
	Normal (averages)†	43.2	0.73	0.15	1.49	89
W. P.‡	Chronic hypochromic anemia	25.5	0.52	0.07	1.84	90
C. O.	" " "	26.0	0.69	0.22	2.04	77
B. A.	" " "	29.0	0.76	0.17	2.20	84
F. J.‡	" " "	30.5	0.65	0.13	1.84	86
A. B.	" " "	35.1	0.71	0.07	1.88	93
L. F.‡	" hyperchromic "	16.4	0.43	0.06	2.32	88
E. K.‡	" " "	29.8	0.58	0.11	1.68	86
E. M.‡	" " "	33.6	0.65	0.10	1.76	91
C. H.	Polycythemia vera	52.0	0.94	0.15	1.67	93
K. K.	" "	55.0	0.89	0.11	1.53	94
H. H.‡	" "	60.0	1.00	0.14	1.57	94
E. E.	" "	60.2	1.00	0.10	1.59	96

* The same explanatory statements, added as foot-notes to these columns in Table III, apply here also.

† See Table III for the individual values averaged to obtain these normal figures.

‡ The blood from these patients was furnished through the kindness of Dr. T. K. Gruber and Dr. R. H. Lyons of the Eloise Hospital, Eloise, Michigan.

total blood value and upon the distribution of the compound was studied. The results are presented in Table IV. When the hematocrit is low, there is no parallel decrease in the total blood nicotinic acid values. This is due to an appreciable increase in the concentration of the nicotinic acid in the corpuscles. When the hematocrit is high, there is a passive increase in the total blood

nicotinic values due simply to the greater number of cells; the concentration of the compound in the corpuscles remains practically in the normal range.

Nicotinic Acid Tolerance Curves—Typical serial blood and plasma[3] values obtained after the administration of test doses of

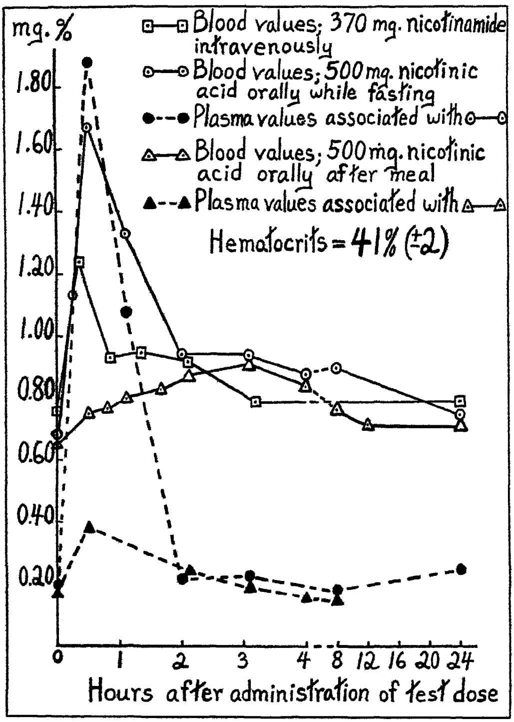

FIG. 1. Influence of the mode of administration of the test dose upon the nicotinic acid tolerance curve. Values for concomitant urinary excretion will be found in Fig. 3 of the preceding paper (14).

nicotinic acid and nicotinamide to the normal subject are plotted in Fig. 1. A difference in the curves is noted when the test dose is administered after eating compared with administration of

[3] Because of the possibility that the erythrocytes *in vitro* can synthesize the nicotinamide-containing coenzymes (13), the bloods were centrifuged immediately after collection to yield the plasma samples for the analyses.

nicotinic acid in the postabsorptive state. In the former case there is a slow increase in the blood values, with the maximum at 3 hours after the test dose is taken. In the latter case the maximal value is not only much greater but also occurs very much sooner—during the first half hour period. The plasma values show an even greater increase but return promptly within 2 hours to the basal range. Practically the same curves were obtained when nicotinamide constituted the test dose. This similarity in blood levels, after nicotinic acid and nicotinamide ingestion, is in marked contrast to the much lower urinary excretion following administration of the latter (14).

The persistence in the elevation of the blood values, after the plasma values returned to normal, is probably due to the union of the nicotinic acid (or amide) in the red blood cell with other compounds to form a complex which does not readily leave the cell. Kohn and Klein (13) have already demonstrated that the erythrocytes both *in vitro* and *in vivo* can synthesize the nicotinamide-containing coenzymes from free nicotinic acid, while von Euler and associates (15) have shown that these compounds are retained in the cells and are not excreted into the urine. It seems likely that a gradual synthesis of the coenzymes occurs when nicotinic acid (or nicotinamide) is taken after eating. This would explain the appearance of the maximal blood value fully 2 hours after the maximal urinary excretion of the compound (14) and after the subsidence of the attendant flushing reactions. Such an interpretation is supported by the correlation of the nicotinic acid curve for plasma (free nicotinic acid) with the urinary excretion and flushing reactions.

We have used nicotinamide for parenteral administration because of the unpleasant side reactions which follow the administration of the desired doses of nicotinic acid. In the experiment plotted in Fig. 1 the amide was injected intravenously at a dose of 5 mg. per kilo of body weight.[4] The relatively small increase in the blood nicotinic acid and the prompt return to the basal level cannot be explained by rapid excretion of the compound; only 10 per cent of the test dose was found in the urine and most of that (94 per cent) as trigonelline.

[4] Such a dose is equivalent to about 9 times the total quantity (free and combined) of nicotinic acid in the blood.

In the curves plotted in Fig. 1 the blood values returned to the basal levels well within 24 hours after single doses. When the normal subject receives *repeated* doses of the compound for an appreciable period of time the elevated blood levels persist long after dosage has ceased. In Fig. 2 are plotted both the blood and plasma values obtained at the same time for a normal subject who received nine 200 mg. oral doses of nicotinic acid, one every 2 hours. The experimental routine is exactly the same as that used

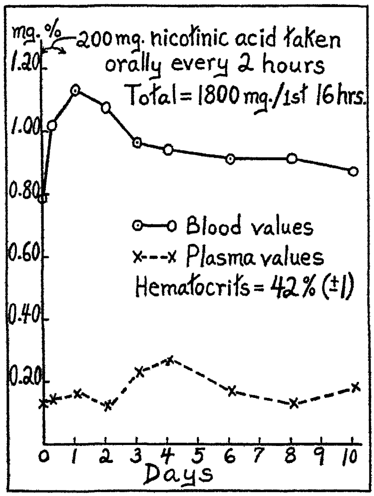

FIG. 2. Persistence in the elevation of the blood nicotinic acid values long after the subject had received repeated oral doses of extra nicotinic acid.

by Axelrod, Gordon, and Elvehjem (16) in following the changes in the coenzyme I content of the blood. They observed an increase in this compound of from 152 to 310 per cent, the peak value occurring 3 days after the first dose. In our experiments with three subjects, the peak value[5] was always associated with the first

[5] For comparative purposes, only the increases in the concentration of nicotinic acid in the corpuscles should be compared with the reported increments of coenzyme I, since the latter is entirely in the erythrocytes. In our experiments there was an average maximal increase of 50 per cent in the corpuscular nicotinic acid concentration.

blood sample taken after dosage ceased. This difference may be due to the determination in our experiments of total nicotinic acid, whereas in theirs only the concentration of one of the nicotinamide-containing coenzymes was estimated. The persistence of the elevated blood values (above 0.90 mg. per cent) is confirmatory of the findings reported by the Wisconsin group (16) and by Kohn (17). The investigations from these other laboratories in which unrelated methods were used have shown the higher blood values to be due to a greater concentration of the nicotinamide-containing coenzymes in the erythrocytes. In our studies, no significant change was found in the plasma values during and immediately following dosage. The only increase occurred during the period coinciding with the marked drop in the nicotinic acid values for whole blood. This was observed in two of the three experimental subjects; its significance requires further study.

SUMMARY

Nicotinamide is stable in blood, stored at 5–8°, for a period of at least 5 days. The values for normal male adults vary from 0.54 to 0.83 mg. per cent, with an average of 0.69 mg. per cent; for female adults the range of values extends from 0.52 to 0.74, with an average of 0.62. Approximately 90 per cent of the total blood nicotinic acid is in the corpuscles. In anemic blood samples the corpuscular nicotinic acid concentration is sufficiently high for the whole blood values to tend to remain in the normal range. In polycythemic bloods, there is a passive increase in the whole blood values due simply to the greater number of cells. A recently ingested meal, coffee drinking, and smoking do not affect appreciably the blood nicotinic acid values. When an oral test dose of nicotinic acid is taken by the fasting subject, there is a prompt increase in the blood values to a maximum, followed by a rapid return to values somewhat above the basal level. These changes are reflected in the urinary excretion figures. When the oral test dose of nicotinic acid is taken after eating, there is a slow increase in the blood nicotinic acid values, the maximum appearing fully 2 hours after the maximal urinary excretion of the compound. The plasma (free nicotinic acid) values and not the whole blood figures coincide with both the extent of the urinary excretion of nicotinic acid and the severity of the unpleasant side reactions

associated with taking nicotinic acid. When the normal subject receives *repeated* doses of the compound, the elevated blood levels persist long after dosage has ceased. There is a similarity in blood levels after nicotinic acid and nicotinamide ingestion; this is in marked contrast to the much lower urinary excretion following administration of the latter. Reproducibility data and recovery experiments with added nicotinamide and coenzyme I support the validity of the method and justify the conclusions drawn.

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THE STRUCTURE OF CEPHALIN*

By E. LeB. Gray

(*From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York*)

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The chemistry of cephalin does not have the satisfactory agreement with theory which characterizes that of lecithin. The identified cleavage products of cephalin differ from those of lecithin only in the type of fatty acid and base present. The work of isolation and identification of these cleavage products has been thoroughly reviewed in the monographs by MacLean and MacLean (1) and Thierfelder and Klenk (2). The formula of cephalin has been arrived at by analogy with the rather well established formula for lecithin. This analogy would be valid if observed data such as elementary analysis and percentage recovery of fatty acids fell into line with the postulated formula as is the case with lecithin, but in most cases they do not. Rudy and Page (3) have obtained evidence of an alcohol-soluble cephalin, analysis of which agrees quite closely with values calculated for the accepted formula. It is the alcohol-insoluble compound, however, to which reference is herein made.

Cephalin appears to behave in a different manner from lecithin both *in vivo* and *in vitro*. It plays an important rôle in blood coagulation (4), while lecithin is totally inactive. It acts as an antioxidant, while lecithin does not (5). Sinclair (6) has shown that there are apparently two types of phospholipid, one which takes part in active metabolism and one which is structural or fundamental. As an example, the phospholipid of the red blood cells is mainly cephalin (probably structural) while that of plasma

* The data in this paper are taken from the thesis submitted to the Division of Graduate Studies of The University of Rochester in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

is for the most part lecithin (probably metabolic). Because of the physiological importance of cephalin, it becomes increasingly important to know the reason for these differences as a possible explanation of its unique biological and chemical behavior.

The first question to be answered is whether the failure of cephalin to give theoretical figures is due to the presence of impurities. It is extremely difficult to purify cephalin, especially since the other accompanying lipids have similar solubilities. Table I shows clearly that small amounts of accompanying lipid impurities are not responsible for the divergence of theoretical from observed figures. Furthermore, by this method of cephalin preparation the other lipid material is removed almost entirely, as well as

TABLE I

Analytical Figures for Cephalin, Observed and Theoretical, Together with Figures for Other Phospholipids and Cerebrosides

Material	C	H	N	P
	per cent	*per cent*	*per cent*	*per cent*
Cephalin, observed*	60.41	9.79	1.67	3.59
" theoretical for stearyl-linoleyl	66.04	10.71	1.88	4.16
Lecithin, stearyl-linoleyl	65.75	10.71	1.74	3.86
Sphingomyelin, lignoceryl	67.72	11.53	3.37	3.88
Cerebroside, nervone	71.13	11.33	1.73	

* Averages from the literature (7–10, 3).

water-soluble impurities which may have been adsorbed on the cephalin molecule.

The amino nitrogen values obtained indicate that a very small amount of other lipids is present. In general, 90 per cent or more of the total nitrogen was in the amino form with a usual variation of 5 per cent and an extreme of 20 per cent.

The second question is whether or not the presence of a group or groups capable of conferring on cephalin its observed properties could be demonstrated; this has been done.

EXPERIMENTAL

Preparation of Cephalin—The method of isolating the cephalin from tissues was based on a method of Bloor (11). Inasmuch as

this procedure was used throughout the investigation, a specimen run will be given. 5 pounds of beef brains were freed of blood vessels and adhering tissues, cut up, run through a meat grinder, stirred up in 6 liters of 95 per cent alcohol, and allowed to stand overnight in the refrigerator. The next morning the mixture was warmed to 50° and filtered. The tissue was then divided into four portions. Each portion was extracted with boiling 95 per cent alcohol for 3 hours. 400 cc. of alcohol were used which was renewed hourly so that each portion was extracted with a total of 1200 cc.

The alcohol extracts, including the original filtrate, were combined and all the alcohol distilled off under reduced pressure, care being taken not to let the temperature rise above 60°. The residue was in the form of a watery emulsion. This was extracted three times with petroleum ether. Moist ethyl ether was occasionally employed because the tendency to form emulsions was much less. The petroleum ether solution was precipitated with 3 volumes of acetone and the precipitate centrifuged off. After removal of the filtrate, the precipitate was scrubbed with acetone until no further coloring matter dissolved in the acetone. The acetone-insoluble material was dissolved in a minimal amount of petroleum ether and placed in the ice box overnight. Any white matter was centrifuged off. The solution was then reprecipitated with acetone, redissolved in petroleum ether, and the white matter allowed to settle out overnight in the cold. Three precipitations with acetone were carried out, followed by three precipitations with absolute alcohol; additional sphingomyelin and cerebrosides were allowed to settle out. After the 6th night of settling in the cold in petroleum ether solution, no more white matter could be obtained. Analysis of this material gave carbon 60.1, hydrogen 8.99, nitrogen 2.04, phosphorus 3.46,[1] amino nitrogen 2.02 (12), ash 12.0, and iodine number 75 (13, 14). The yield was 44 gm.

Brain cephalin was a very hygroscopic, light colored powder, which darkened and quickly became sticky on exposure to air and moisture. Samples had most of their nitrogen in the amino form and gave 60 to 70 per cent fatty acids. The iodine number ranged from 65 to 100. Both beef heart and liver gave compounds

[1] The microanalyses were carried out by Dr. L. T. Hallet of the Kodak Research Laboratories.

which, compared to brain cephalin, were much more highly colored; their phosphorus was much higher and, as would be expected, their fatty acid content was much lower.

The occurrence of cephalin split-products in the liver cephalins was shown by a simple fractionation. Upon purification of the liver cephalin by emulsification with water, precipitation with hydrochloric acid, and shaking out with petroleum ether, a material was obtained (see Table II, Liver Fraction I) which differed markedly from the original material. On acetone precipitation

TABLE II

Comparison of Analytical Figures of Cephalin from Various Sources. Effects of Various Procedures on Analytical Data

Material	C	H	N	P	I No.	Fatty acids	Ash
	per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*
Heart	55.9	8.23	1.55	4.12	65.3		
Liver	51.52	7.43	1.88	3.31	69.1	59.0	
" Fraction I	57.32	8.18	1.25	3.20	90.3	69.0	
" " II	51.8	7.43		4.23	38.6	43.4	
Brain, original	60.1	8.99	2.04	3.46	75.0		12.0
" treated	62.0	9.39	1.85	2.93			
" acetone-pptd.	60.0	9.12	2.08	3.38			
" hydrolyzed	73.26	10.83	0.44				
Calculated for oleic acid	76.95	12.07					
Brain, reduced	60.65	9.15	1.75	2.97	27.0		13.4
" reprecipitated	61.10	9.85	1.14	3.89			
Calculated for distearyl cephalin, $C_{41}H_{82}NPO_8$	65.73	10.98	1.87	4.15		76.0	

of the emulsified cephalin, another fraction (see Table II, Liver Fraction II) was obtained which differed both from Liver Fraction I and from the original.

To produce any change in brain cephalin by this procedure, it must be sufficiently rigorous to hydrolyze the material partially. For example, the sample of brain cephalin whose analytical figures have already been given was emulsified with water and then precipitated by the addition of 3 to 4 volumes of water and a little hydrochloric acid. A sample of this material was then shaken for 43 hours with acidulated (5 per cent HCl) water. It was then filtered, scrubbed with acetone, and dissolved in petroleum ether

(see Table II, brain, treated). Upon precipitation with acetone, the analytical figures were almost identical with the original material (see Table II, brain, original *versus* acetone-precipitated). Thus the portion of this material which had not been hydrolyzed was unchanged. There was no fractionation, as occurred in liver and also in heart cephalins. The petroleum ether-soluble portion (Table II, brain, hydrolyzed) gave a phosphorus-free material with negligible nitrogen content of which the carbon and hydrogen values are like those for the higher fatty acids; for example, those of oleic acid which are included in Table II for comparison.

Reduced Cephalin—3 gm. of cephalin from the brain were dissolved in 100 cc. of a mixture of half and half cyclohexane and glacial acetic acid. 0.2 gm. of Voorhees and Adams' platinum oxide catalyst (15) was added and the reaction vessel continuously shaken until the solution no longer took up hydrogen. This took about $7\frac{1}{2}$ hours. The solution was heated to dissolve the reduced cephalin completely and to coagulate the catalyst. The solution was filtered hot. This material had an iodine number of 5.2.

A second batch of 9.79 gm. of cephalin with 0.55 gm. of catalyst was allowed to reduce for 3 days. At this time the reaction was stopped and the material filtered. It was not completely reduced (iodine number 27), although in its physical properties it was similar to the other sample. A fraction was obtained by reprecipitation from hot chloroform with methyl alcohol which gave increased phosphorus and decreased nitrogen values (see Table II). The calculated figures for distearyl cephalin are included for comparison.

Reduced cephalin is a white, non-hygroscopic material unlike the synthetic saturated cephalin of Grün and Limpächer (16) and of Kabishima (17), which in both cases is referred to as being quite hygroscopic. It is much less soluble than the unreduced compound, although in its composition no great differences could be detected. It is soluble in cyclohexane, toluene, hot glacial acetic acid, and hot chloroform; somewhat soluble in ether and petroleum ether; and insoluble in alcohol and acetone. Having no double bonds, it is exceedingly stable. It melts between 156–162°. It is, however, amorphous, as shown by its x-ray diffraction pattern. In water-clear chloroform solution, it gives a Tyndall effect.

While the existence of more than one type of cephalin has been

demonstrated (3, 18), in the classical, alcohol-insoluble cephalin more groups than have been already isolated can be demonstrated. The additional group or groups do not appear in the fatty acid fraction, which is already low. Therefore, they must be sought in the water-soluble fraction of the molecule. This hypothesis is strengthened by the ultimate analyses of the compound, since the missing portion must be low in carbon and hydrogen, rich in oxygen, and therefore probably water-soluble.

The water-soluble fraction was first tested with qualitative sugar reagents. When cephalin is hydrolyzed with dilute sulfuric acid, the water layer may or may not reduce alkaline copper reagents. However, if the solution is allowed to stand for a sufficient time, it will always reduce these reagents. For this reason the presence of sugars was at once suspected. The water-soluble hydrolysate was strongly positive with the hot iodoform reaction and with Fehling's solution. Positive reactions were obtained from α-naphthol, thymol, and Benedict's solution. Orcinol (Bial's test) once gave a positive test on standing but was usually negative. The cold iodoform reaction was positive. Naphthoresorcinol (Tollens' reagent) gave a somewhat atypical positive test for glucuronic acid. Aniline acetate paper (furfural test) was positive on 5 to 10 minutes heating with concentrated HCl. Codeine phosphate (Denigès' test for glucuronic acid (19)) was positive. On the other hand, negative results were obtained from the resorcinol (Seliwanoff's test), *p*-bromo- and *p*-nitrophenylhydrazine, thiosemicarbazide, and the mucic acid test. Phenylhydrazine gave an unidentifiable precipitate.

Although simple sugars must be absent, quantitative measurements of reduced copper (Bertrand) gave values from 2 to 5 per cent calculated as glucose, instead of the expected 18 per cent, indicating that the substance responsible for the reduced copper was not the primary compound, but the result of a secondary reaction.

The reactions with orcinol, aniline acetate, naphthoresorcinol, and codeine sulfate made glucuronic acid a possibility. However, no osazone could be obtained. An attempt was then made to measure glucuronic acid quantitatively according to the method of Dickson, Otterson, and Link (20). The solution of cephalin was treated with 12 per cent HCl for 5 hours at 135° and the

CO_2 collected in a Truog absorption tower by means of $Ba(OH)_2$. The excess hydroxide was titrated and the CO_2 calculated from the amount of $BaCO_3$ formed. 5 per cent CO_2 should have been found, assuming a uronic acid to be present. Actually in four runs 0.85, 1.23, 0.95, and 1.52 per cent, respectively, were found. Obviously there is no mole to mole relationship. This, taken with the fact that no osazone could be isolated, led to the exclusion of glucuronic acid.

In every case, the presence of glycerophosphoric acid interfered with the isolation of a derivative when attempts were made to form salts of brucine, cinchonine, and quinine, as well as barium, calcium, zinc, and lead. On one occasion a brown, caramel-like substance was isolated. This substance was soluble in water, insoluble in alcohol, reduced Fehling's solution, and was acid to litmus. It could not be further resolved. Small amounts of calcium and zinc salts were isolated, but in insufficient quantities to give a clue as to the identity of the acids. *p*-Bromophenacyl bromide gave no crystalline derivative.

An acid-water solution, giving a strong reduction with Fehling's solution, was treated with copper sulfate and calcium hydroxide, to remove all reducing substances. Following the procedure for determining lactic acid (21, 22) a veratrole test was made which was positive. Attempts to isolate the aldehyde with dimethyldihydroxycyclohexane gave a precipitate but too little for identification, nor could a derivative be obtained with either phenylhydrazine or its *p*-nitro derivative.

DISCUSSION

Cephalin, after care has been taken to exclude all known substances likely to occur as contaminants, has essentially the same elementary analysis as has been reported by other workers. This can only mean that some unknown substance, rich in oxygen and free from nitrogen and phosphorus, must be present, either as an impurity extremely difficult to remove, or as a part of the molecule of cephalin.

Any contamination from other lipids would not cause the curious lowering of all analytical figures simultaneously. Lecithin can be obtained with figures which correspond quite closely to its formula, whereas cephalin figures are always lower than those for

lecithin (Table III). Oxidation does not account for this phenomenon, for (1) the fatty acid percentage is always too low and (2) the reduced cephalin shows the same discrepancy. Cuorin is not responsible, for it occurs preformed in liver and heart but not in brain. Furthermore, it is not formed during the isolation of the cephalin, since the most rigorous treatment given brain cephalin, lengthy shaking with 5 per cent HCl, although sufficiently severe to hydrolyze the molecule, failed to form any trace of cuorin. This points to the presence of an additional unknown compound, rich in oxygen. Furthermore, this oxygen-rich compound is of the hydroxy acid type.

Evidence for this type of compound has accumulated. Thus, if the acid hydrolysate from cephalin is examined, it is found to

TABLE III

Comparison between Observed and Theoretical Analytical Figures for Cephalin and Those for Lecithin

Material	C	H	N	P
	per cent	*per cent*	*per cent*	*per cent*
Cephalin, found (brain)..........	60.1	8.99	2.04	3.46
Calculated for stearyl-linoleyl...	66.04	10.71	1.88	4.16
Lecithin, found (23)............	65.39	10.49	2.07	3.93
Calculated for stearyl-linoleyl...	65.75	10.71	1.74	3.86

contain a reducing substance; the reduction increases on standing. If the reducing substance responsible is removed with a lime-copper sulfate mixture, more can be regenerated by treating with cold, concentrated H_2SO_4, suggesting a compound similar to lactic acid. This idea that the reducing compound is the result of a secondary reaction and is not the primary compound in the water layer is further emphasized by the fact that CO_2 produced by the treatment of cephalin with HCl, after the manner of Link, is such as to indicate the result of a secondary reaction.

SUMMARY

1. Cephalin, after care has been taken to exclude all known substances likely to occur as contaminants, has essentially the same elementary analysis as has been reported by other workers.

2. The discrepancy between calculated and actual analytical figures may be accounted for by the presence of an additional

group or groups, low in carbon and hydrogen, rich in oxygen. Evidence for the presence of such groups has been demonstrated.

3. Cuorin is not formed during the process of lipid extraction, but exists preformed. It is present in heart and liver, but not in brain.

4. Reduced cephalin does not differ from unreduced cephalin except in such physical and chemical properties as are inherent in its saturation. It is non-hygroscopic.

The author wishes to thank Professor Walter R. Bloor and Professor Harold C. Hodge for criticism and advice.

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THE SYNTHESIS OF FLAVIN-ADENINE DINUCLEOTIDE FROM RIBOFLAVIN BY HUMAN BLOOD CELLS IN VITRO AND IN VIVO

By J. RAYMOND KLEIN AND HENRY I. KOHN

(*From the Departments of Biochemistry and of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina*)

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Riboflavin, which is a vitamin for man (1), various lower animals (2–5), and bacteria (6), has been isolated from biological material in three forms, flavin nucleoside, *i.e.* riboflavin (7), flavin mononucleotide (8, 9), and flavin-adenine dinucleotide (10). The mononucleotide in combination with a specific protein forms a pyridine nucleotide oxidase (8, 11, 12). The dinucleotide consists of the mononucleotide combined with adenylic acid, and when joined to various specific proteins forms the *d*-amino acid oxidase (10), the xanthine oxidase (13), and pyridine nucleotide oxidases (14–16). Since the dinucleotide has more functions and a wider distribution, it has been suggested that the mononucleotide is derived from it in preparation (14).

Flavin-deficient diets decrease the tissue level of riboflavin in the dog (17) and rat (18–20), and of flavin-adenine dinucleotide in the rat (21). Rapid restoration of the dinucleotide level follows the administration of riboflavin to deficient animals (21).

The object of the present work is to furnish data indicating that the cells of normal human blood can synthesize flavin-adenine dinucleotide from riboflavin *in vitro* and *in vivo*. In addition, the fate of ingested riboflavin, as measured by urinary excretion, has been studied.

EXPERIMENTAL

Assay of Flavin-Adenine Dinucleotide—The method is based upon the alanine test of Warburg and Christian (10). Variations of the method have been used to measure the dinucleotide concen-

tration of animal tissues (21, 22), and in the purification of the pyridine nucleotide oxidase (16) and the specific protein of the *d*-amino acid oxidase (23).

The procedure used in the present study was as follows: 0.2 ml. of cells or plasma was added to 1 ml. of water in the well of a Warburg vessel. The vessel was immediately placed in a water bath at 95–98° and after 3 minutes was cooled in cold water. 0.8 ml. of 0.1 M pyrophosphate buffer, pH 8.3, was added, and the contents of the vessel stirred with a rod. Then 4 mg. of a preparation of the specific protein of the *d*-amino acid oxidase dissolved in 0.3 ml. of water were placed in the well, and 0.2 ml. of 0.64 M *dl*-alanine in pyrophosphate buffer in the side arm. Alkali was put in the inset. The vessel was filled with oxygen. After temperature equilibration at 37.5° the alanine was tipped into the well, and the rate of oxygen uptake measured during a 30 minute period. The amount of dinucleotide in the material was obtained by a comparison of the rate it produced with the rates produced by known amounts of the dinucleotide.

Duplicate determinations were run in all cases. The difference between duplicates in the assay of 0.2 ml. of cells was never more than 5 per cent, and in the assay of 0.2 ml. of plasma, 25 per cent. Nucleotide, in amounts from 0.03 to 0.15 γ, added to the water before the material to be tested, or after heating, could be determined with an error of not more than ± 5 per cent. The rate and extent of oxidation of 0.5 and 1 mg. of *d*(−)-alanine in the presence of excess dinucleotide (80 γ) were not affected by the presence of 0.2 ml. of heated cells or plasma. These experiments show that there is no substance in heated blood which affects the oxidation of *d*(−)-alanine by the *d*-amino acid oxidase.

The assay was not changed when the period of heating the diluted sample varied from 2.5 to 4 minutes, or when the diluted sample was allowed to stand 4 minutes before heating. In addition, the assay was not changed by adding the material directly to hot water. 1 to 2 mg. of potassium oxalate per ml. of blood did not affect the assay of cells or plasma.

That the substance in the cells giving the alanine test is flavin-adenine dinucleotide is indicated by the test itself and by the following. 100 ml. of cells were mixed with 100 ml. of water. Trichloroacetic acid (40 gm. per 100 ml.) was added until no more

precipitate was formed. The filtrate from this mixture was adjusted to approximately pH 6. 1 gm. of ammonium sulfate was added per 2 ml. of solution. The mixture was extracted three times with 3 ml. portions of liquefied *p*-cresol. The cresol extract was diluted with 2 volumes of ether and extracted several times with small portions of water. The color of the extract was yellow. It gave the alanine test. The extract was made just acid to Congo red paper with nitric acid. Silver nitrate was added. The precipitate formed was collected and decomposed with hydrogen sulfide. The filtrate, which had a yellow color, gave the alanine test. No attempt was made to make these experiments quantitative. They indicate, however, that there is a substance in human blood cells that can be extracted with cresol, precipitated with silver nitrate in acid solution, and function in the alanine test. Flavin-adenine dinucleotide has these properties (10).

The specific protein was prepared from pig kidney by the method of Warburg and Christian (10). The amount and purity of the dinucleotide standard, prepared from bakers' yeast as the barium salt (10, 14), were estimated with the aid of the Coleman photoelectric spectrophotometer equipped with a 10 mμ slit and square cuvettes. The barium salt, at a concentration of 12.8 γ per ml., had an extinction coefficient[1] of 0.167 at 450 mμ. Pure riboflavin (Merck), concentration 10 γ per ml. or 2.66×10^{-5} M, had an extinction coefficient of 0.437 at 450 mμ. Since riboflavin and flavin-adenine dinucleotide have the same molar extinction coefficient[2] at 450 mμ (10), the calculated concentration of dinucleotide was $0.167/0.437 \times 2.66\ 10^{-5}$ M. The molecular weight of the barium salt is 920. The calculated concentration of the barium salt was 9.34 γ per ml. The purity was therefore $9.34/12.80 = 0.73$.

The absolute values reported in this work depend upon the purity of the standard. The relative values and the conclusions derived from them are independent of the purity.

Assay of Riboflavin in Urine—By the method employed the increment in riboflavin excretion induced by dosage with the

[1] The extinction coefficient is the logarithm to the base 10 of the reciprocal of the transmission, the diameter of the cell being taken as unity. It is proportional to the concentration.

[2] The molar extinction coefficient is the extinction coefficient of a molar solution.

vitamin can be measured. This extra riboflavin was determined with the aid of the Coleman photoelectric spectrophotometer equipped with a 10 mμ slit and round cuvettes. The theory of the determination, based upon standard spectrophotometric principles, is as follows:

For a sample of urine containing extra riboflavin, let

(1) $E_1 = R_1 + U_1 =$ extinction coefficient of the sample at wave-length 1
(2) $E_2 = R_2 + U_2 =$ extinction coefficient of the sample at wave-length 2

where R_1 and R_2 are the extinction coefficients of the extra riboflavin, and U_1 and U_2 the extinction coefficients of the urine alone at wave-lengths 1 and 2 respectively. Let

$$(3)\quad \frac{R_1}{R_2} = K_R \qquad \text{and} \qquad (4)\quad \frac{U_1}{U_2} = K_U$$

From Equations 1 to 4 it follows that

$$(5)\qquad R_1 = \frac{K_R}{K_R - K_U}(E_1 - E_2K_U)$$

The concentration of extra riboflavin in the urine is given by

$$(6)\qquad \text{Concentration of extra riboflavin} = R_1 \times A$$

where A is the concentration of riboflavin per unit of extinction coefficient.

The protocol in Table I contains the data of a typical determination: E_1 and E_2 were obtained from measurements made upon a given sample of urine containing extra riboflavin. K_R and A, which are constants, were obtained from measurements made upon solutions of riboflavin in water. The measurements needed to establish K_U cannot be made upon a sample of urine containing extra riboflavin.

The K_U values used were obtained from measurements made upon urine collected before the ingestion of riboflavin. This involves the assumption that K_U is a constant for a given individual during the period of experimentation. This assumption seems justified, because the K_U value for a given individual was relatively constant from day to day and for different periods of the same day. In addition, a change in K_U produces a relatively smaller change in the calculated concentration of extra riboflavin. In the example given in Table I an increase in K_U from 0.40 to

0.50, *i.e.* of 25 per cent, would decrease the calculated concentration from 0.037 to 0.035 mg. per ml. or 5 per cent. The maximum difference in the K_U values for the urines of the individuals studied was 0.05.

445 mμ was chosen as wave-length 1, because riboflavin has its maximum absorption at this wave-length. Wave-lengths from 370 to 400 mμ were found suitable for wave-length 2; 390 mμ was chosen. All urines were adjusted to pH 5.1 to 5.3 before the optical measurements were made. Marked variations in pH, *e.g.* 3 and 8, produce a difference in the extinction coefficients of

TABLE I

Determination of Extra Riboflavin in Urine

Sample	Transmission		Extinction coefficient	
	445 mμ	390 mμ	E_1 445 mμ	E_2 390 mμ
Control urine diluted 1:5	0.648	0.374	0.187	0.426
Urine containing extra riboflavin diluted 1:5	0.350	0.410	0.455	0.387
Riboflavin in water, concentration 0.01 mg. per ml.	0.280	0.431	0.553	0.365

$$K_R = 0.553/0.365 = 1.51 \quad A = 0.01/0.553$$
$$K_U = 0.187/0.426 = 0.44 \quad R_1 = 0.402$$

$$\text{Concentration of extra riboflavin in urine} = 0.402 \times \frac{0.01}{0.553} \times 5 = 0.0363 \text{ mg. per ml.}$$

urine, although K_U is not changed appreciably. After the pH was adjusted, the urines were suitably diluted, and the measurements made.

Riboflavin added to urine to produce an extra concentration of 0.001 mg. per ml. can be estimated with an error of ±15 per cent. Urine containing 0.0001 mg. per ml. cannot be estimated with any reasonable accuracy. The deviation of the calculated value from the theoretical becomes less with increasing concentration of added riboflavin; *e.g.* ±5 per cent at 0.01 mg. per ml.

Synthesis of Flavin-Adenine Dinucleotide in Vitro—Defibrinated blood to which Ringer-phosphate solution or Ringer-phosphate solution containing riboflavin has been added was incubated in

stoppered test-tubes at 30–34°; sterile technique was employed. After various intervals the material was centrifuged, the cells and plasma separated, and aliquots of each were analyzed by the procedure described. The experimental details and the results are given in Table II.

TABLE II

Effect of Incubation with Riboflavin upon Flavin-Adenine Dinucleotide Concentration of Human Blood Cells and Plasma

2 ml. aliquots of defibrinated blood plus either 0.1 ml. of Ringer-phosphate solution (control) or 0.1 ml. of Ringer-phosphate solution containing riboflavin (experimental) were incubated at 30–34°. At the times indicated 0.2 ml. of cells and plasma was assayed.

100 γ of riboflavin were added in Experiments 1, 2, and 3; 1 γ in Experiment 4. The same sample of blood was used in Experiments 3 and 4. The blood used in Experiments 1, 2, and 3 was obtained from different individuals.

Experiment No.	Incubation period	Cells				Plasma	
		Control	Experimental			Control	Experimental
		Flavin-adenine dinucleotide	Flavin-adenine dinucleotide	Change over initial value	Change over control	Flavin-adenine dinucleotide	Flavin-adenine dinucleotide
	hrs.	*γ per ml.*	*γ per ml.*	*per cent*	*per cent*	*γ per ml.*	*γ per ml.*
1	0	0.75	0.75			0.10	0.12
	5	0.72	0.93	24	29	0.10	0.10
	11	0.69	0.96	28	39	0.10	0.10
	21	0.57	0.69	−8	21	0.08	0.08
2	0	0.75	0.79			0.10	0.11
	6	0.67	0.85	8	27	0.09	0.11
	11	0.58	0.72	−9	24	0.08	0.09
	24	0.66	0.74	−6	12		
3	0	0.71	0.71				
	24	0.64	0.81	14	27		
4	0	0.71	0.71				
	24	0.64	0.75	6	17		

The data in Table II show that a substance giving the alanine test was formed in human blood cells incubated with riboflavin; presumably it was flavin-adenine dinucleotide. The fall in the plasma level, which was not affected by the addition of riboflavin, was not of sufficient magnitude to account for the increase in

the cells. The initial values obtained in the present study are compatible with those found for ox and rat blood (21).

From an analytical view-point it is of interest to note that the concentration of dinucleotide in the cells and plasma decreases with time, and that the presence of riboflavin does not affect the assay.

Synthesis of Flavin-Adenine Dinucleotide in Vivo—A control sample of blood was taken, after which the subject drank a suspension of riboflavin in water. No reactions of any kind were noted. At various times thereafter blood samples were taken for analysis, and more riboflavin ingested. The results are presented in Table III.

The data in Table III, Experiments 1 and 2, indicate that the level of flavin-adenine dinucleotide in normal human blood cells and plasma is fairly constant. In view of the constancy of the normal level, the data show that the ingestion of riboflavin produced an increase in the flavin-adenine dinucleotide concentration of the cells, but not of plasma. The increase was of the same order of magnitude as was obtained *in vitro*. The results *in vivo* and *in vitro* are in agreement, and indicate that the same process occurs in both cases.

Assuming a cell volume of 3000 ml. for the subject of Experiment 3, Table III, it can be calculated that 0.32 mg., or 0.14 per cent, of the ingested riboflavin (226 mg.) was recovered in the cells as the dinucleotide. *In vitro*, in Experiment 4, Table II, 1 γ of riboflavin was added to blood, of which 0.054 γ, or 5 per cent, was recovered as the dinucleotide. Increasing the concentration of riboflavin 100 times increased the amount of dinucleotide recovered to 0.082 γ.

3 or 4 days after the ingestion of riboflavin had been discontinued, the level of the dinucleotide returned to normal.

In other experiments, in which 0.1 ml. of finger blood was used for assay, similar results were obtained.

Excretion of Extra Riboflavin in Urine—Urine was collected during the course of Experiments 1, 2, and 3, Table III, and the extra riboflavin content determined by the method described. The data for Experiments 1 and 3 are presented in Fig. 1.

The data in Fig. 1 show that a larger percentage and absolute amount of the riboflavin were excreted following successive doses,

particularly in the case of the subject of Experiment 1. The differences in the percentage and total excretion represented by Curves 1 and 4 indicate a difference in the original degree of

TABLE III

Effect of Oral Ingestion of Riboflavin upon Flavin-Adenine Dinucleotide Concentration of Human Blood Cells and Plasma

5 ml. of venous blood were taken in 5 mg. of potassium oxalate at the times indicated. The blood was centrifuged 25 minutes at 2000 R.P.M. 0.2 ml. aliquots of cells and plasma were analyzed by the procedure described. The same subject was used in Experiments 1 and 2. The first assay in Experiment 2 was made 42 days after the last assay of Experiment 1; the second assay in Experiment 2 was made 1 day later.

Experiment No.	Amount of riboflavin ingested	Time	Flavin-adenine dinucleotide		
			Cells		Plasma
			Assay	Increase	Assay
	mg.	*hrs.*	*γ per ml.*	*per cent*	*γ per ml.*
1	100	0	0.81		0.14
	50	12.6			
	50	22.2			
	10	24			
		35	0.95	17	0.11
		123	0.75	−7	0.10
2		0	0.83		0.13
	50	0	0.79		
	50	8			
	50	16			
	50	24	0.97	23	0.12
		30	0.92	16	
		90	0.83	5	
		180	0.80	1	0.14
3	100	0	0.74		0.14
	13	12.5			
	13	13.5			
	100	24.3			
		29	0.96	30	0.11
		80	0.85	15	
		192	0.75	1	0.14

saturation of the tissues of the individuals studied. Of the 210 to 226 mg. of riboflavin taken by the subjects, approximately 20 to 25 per cent was recovered in the urine. In Experiment 2 22 per cent of the ingested riboflavin was recovered in the urine.

During the periods of maximum excretion, 0.5 ml. of urine and 0.5 ml. of saliva were tested for dinucleotide. If any was present, its concentration was less than 0.005 γ per 0.5 ml.

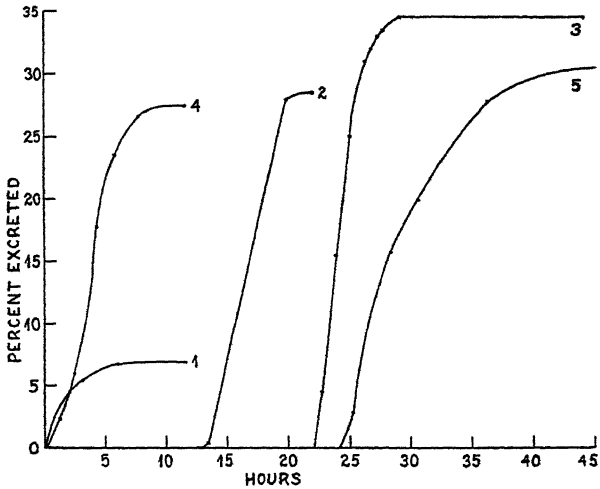

FIG. 1. Renal excretion of riboflavin taken by mouth. The data presented by Curves 1, 2, and 3 were obtained with urine collected during the course of Experiment 1, Table III; Curves 4 and 5, with urine collected during Experiment 3. The curves represent the percentage of the ingested riboflavin that was excreted in the urine as a function of time. *Curve 1*, 100 mg. of riboflavin were ingested at 0 hour. At 12.6 hours the excretion stopped. The total excretion was 6.9 mg. The maximum rate of excretion, 2.2 mg. per hour, occurred during the 2nd hour of this period. *Curve 2*, 50 mg. more of riboflavin were ingested at 12.6 hours. The concentration fell to 0.03 mg. per ml. at 22 hours. The total excretion was approximately 14 mg. *Curve 3*, 50 mg. more of riboflavin were ingested at 22.2 hours, and 10 mg. at 24 hours. The portion of the curve from 22.2 to 24 hours is calculated on the basis of the 50 mg. dose, the rest of the curve on the basis of a 60 mg. dose. At 29 hours the excretion had stopped. The total excretion was 21 mg. The maximum rate of excretion, 5.9 mg. per hour, occurred during the 2nd hour of this period. *Curve 4*, 100 mg. of riboflavin were ingested at 0 hour. The concentration fell to 0.03 mg. per ml. at 12.5 hours. The total excretion was approximately 28 mg. The maximum rate of excretion, 6.0 mg. per hour, occurred during the 3rd hour of this period. 13 mg. more of riboflavin were ingested at 12.5 hours and 13 mg. at 13.5 hours. The excretion stopped at 24 hours. *Curve 5*, 100 mg. more of riboflavin were ingested at 24.3 hours. The excretion stopped at 45.2 hours. The total excretion was 30.3 mg. The maximum rate of excretion, 4.6 mg. per hour, occurred during the 4th hour of this period.

That the substance excreted in the urine was riboflavin or a product of practically identical properties is indicated by the following experiments. (*a*) The substance could not be extracted from acidified urine with chloroform; therefore it is not allied to lumiflavin. (*b*) After illumination in alkaline solution a substance could be extracted with chloroform from acidified urine; the optical properties of the chloroform extract were similar to

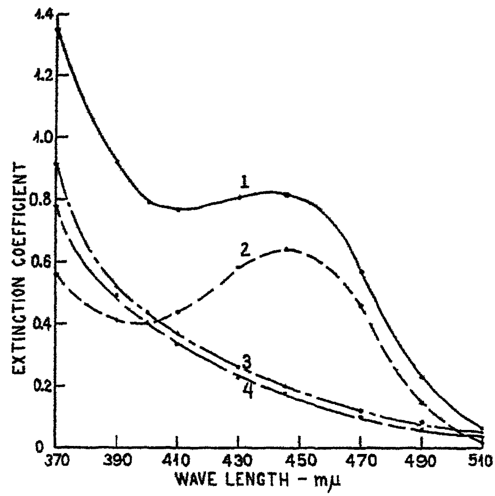

FIG. 2. Riboflavin in urine. The curves represent the extinction coefficients of the solutions, diluted 1:5, at various wave-lengths. *Curve 1*, urine containing extra riboflavin. The calculated concentration of extra riboflavin in the undiluted sample was 0.0115 mg. per ml. *Curve 2*, extra riboflavin. The concentration of the solution was 0.0115 mg. per ml. *Curve 3*, urine alone. This is the curve of the sample of urine minus the extra riboflavin. *Curve 4*, control urine.

those of lumiflavin. (*c*) Phenol extracts of urine had a yellow color, which, following the addition of ether, could be extracted with water. The aqueous extract had optical properties similar to those of riboflavin. The optical properties of the preparations could not be described exactly, because urinary pigment is extracted under the same conditions. The presence of lumiflavin in the first preparation (*b*) and of riboflavin in the second (*c*) was indicated by the presence of maxima at approximately 370 and

450 mμ. (*d*) The extinction coefficient of a urine containing extra riboflavin is the sum of the extinction coefficients of the riboflavin and the urine alone. If the extinction coefficients of the calculated concentration of riboflavin at various wave-lengths are subtracted from the extinction coefficients of the mixture, the extinction coefficients of the urine alone are obtained. Data prepared in this way are given in Fig. 2. Comparison of the forms of Curves 3 and 4, Fig. 2, shows that the pigment of a control urine and the one containing riboflavin are the same. This indicates that the substance measured has the optical properties of riboflavin.

DISCUSSION

The results obtained in the present work indicate that the level of flavin-adenine dinucleotide in human blood cells is increased by the ingestion of riboflavin. Since the phenomenon can be obtained *in vitro,* the synthesis must occur in the blood cells. When the administration of riboflavin is stopped, the level returns to its original value. Although the dinucleotide occurs in plasma, its level in the plasma is not affected by incubation with riboflavin.

These results may be contrasted with those obtained in the synthesis of factor V (pyridine nucleotides) from nicotinic acid by human blood cells (24). The factor V concentration, in terms of diphosphopyridine nucleotide, is approximately 100 times that of the flavin dinucleotide. The increase in the concentration of factor V produced by the incubation of cells with nicotinic acid is of the order of 100 per cent, a larger synthesis than was found in the present experiments. The increase in factor V is accompanied by an increase in the ability of the cells to oxidize lactate in the presence of methylene blue. This is due, presumably, to an increase in the concentration of diphosphopyridine nucleotide. Flavin-adenine dinucleotide is a component of the diphosphopyridine nucleotide oxidase. The increase in the concentration of the flavin dinucleotide, however, is not accompanied by an increase in the ability of the cells to oxidize lactate and glucose in the presence of methylene blue. This shows that the concentration of the dinucleotide is not limiting in the oxidation of these substrates.

The synthesis of diphosphopyridine nucleotide by the cell may be pictured as the conversion of nicotinic acid to the amide and

then the condensation of the amide, ribose, and phosphoric acid with adenylic acid. The synthesis of flavin-adenine dinucleotide would involve the condensation of riboflavin and phosphoric acid with adenylic acid. Since in animal experiments the alloxazine component of the flavin apparently cannot serve as a vitamin (25, 26), it may be suggested that the cell can couple ribose with pyridine nitrogen but not with alloxazine nitrogen.

No function can be ascribed to the flavin or pyridine nucleotides of the blood cells on the basis of available data. The ability of the cells to synthesize and store the nucleotides may indicate that the cells serve to limit the loss of the vitamins by renal excretion.

SUMMARY

1. Human blood cells can synthesize flavin-adenine dinucleotide from riboflavin *in vitro* and *in vivo*.

2. After the ingestion of approximately 200 mg. of riboflavin by mouth, approximately 25 per cent was recovered in the urine as riboflavin and 0.14 per cent in the cells as flavin-adenine dinucleotide. The increase in the dinucleotide concentration in the blood cells was approximately 30 per cent.

3. The concentration of flavin-adenine dinucleotide in urine and saliva after the ingestion of riboflavin is less than 0.005 γ per 0.5 ml.

We are indebted to Merck and Company, Inc., for a supply of pure riboflavin.

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A MICROCOLORIMETRIC METHOD FOR THE DETERMINATION OF POTASSIUM IN BIOLOGICAL MATERIALS

By PETER WALDEMAR SALIT

(From the Department of Ophthalmology, College of Medicine, State University of Iowa, Iowa City)

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Several methods for the determination of potassium in biological materials have been described. According to the principal chemical reactions the most widely used methods may be classified into three groups, methods based on (1) the sodium cobaltinitrite reagent, (2) the silver cobaltinitrite reagent, and (3) the chloroplatinic acid reagent. Certain drawbacks in the first two methods lie in the fact that both the potassium sodium cobaltinitrite and the silver potassium cobaltinitrite compounds are said to be rather doubtful in composition (1–6). The ratio of sodium or silver to potassium is believed to vary according to their relative concentrations in the sample and reagent, the temperature and rate at which the precipitation takes place, and the temperature at which the precipitate is dried. The interfering substances are iron, aluminum, manganese, barium, calcium, magnesium, and copper (7). In principle, the chloroplatinate methods are by far superior to those based on the sodium or silver cobaltinitrite compound, because K_2PtCl_6 has always a definite composition (8–11). Also, in this method, the number of interfering substances is smaller. Ammonium, alcohol, iron, copper, and ferrocyanide are said to interfere (9, 10). But since ashing is always employed with this method, ammonium and alcohol are destroyed, and iron and copper are converted into insoluble oxides in the process. However, the hitherto employed methods of ashing biological materials for potassium determinations are rather complicated. Shohl and Bennett (10) and many others have found loss in potassium by the usual dry ashing procedure. Shohl and Bennett, therefore, first digest the sample with sulfuric acid, and then transfer it to a

platinum crucible for reashing and to drive off the sulfuric acid. Kramer (12) tries to avoid the loss of potassium by ashing in a platinum dish contained in a quartz dish. It is evident that these methods of ashing are too cumbersome, especially when many determinations must be made. Strauss (13) recommends the use of thorium nitrate to facilitate the ashing, but even with this aid the ashing is carried out at a temperature varying between 600–750°, a temperature at which loss of potassium is unavoidable. Moreover, in all potassium determinations in which ashing is employed, the ash must be subsequently transferred by washing into some other container, and frequently, after the solution has been evaporated to dryness, several further transfers combined with filtration are necessary. Under these complicated manipulations a loss of potassium is unavoidable, especially when one is working with very small amounts.

A procedure therefore was devised in connection with the chloroplatinate method that obviates all the drawbacks connected with the methods of ashing and transfer hitherto employed.

In this method the material is ashed in specially made nickel centrifuge tubes at a temperature of 465° ± 10° in the presence of an oxidizing agent, the red mercuric oxide. This is done best in a muffle furnace equipped with a temperature control, *i.e.* thermostat. The potassium in the ash is determined by Shohl and Bennett's (10) colorimetric method.

Reagents and Appliances—

1. A 10 per cent chloroplatinic acid solution, filtered and kept in the ice box.

2. Standard potassium chloride solution. (*a*) A stock solution consisting of 0.7422 gm. of KCl dissolved in 100 cc. of distilled water and kept in an ice box. 1 cc. of this solution contains 4 mg. of K. (*b*) A dilute standard KCl solution for immediate use made up of 5 cc. of the stock solution diluted to 200 cc.; 1 cc. of this solution contains 0.1 mg. of K. The solution is kept in the ice box.

3. Half saturated (72 per cent) potassium iodide solution in a brown bottle. This keeps for about 6 months in a dark, cold place.

4. 1 N HCl or H_2SO_4.

5. Absolute alcohol.

6. A 3 per cent potassium-free gelatin solution. In connection

with ashing, gelatin is added to such samples as contain very little organic matter; *e.g.*, urine and small samples of whole blood, also aqueous and vitreous humors, spinal fluid, and ascitic fluid. Its purpose is to prevent occlusion by inorgainc matter in the process of incineration and thus insure a more thorough ashing. The analysis of 0.8776 gm. of flake gelatin proved the latter to contain no potassium.

7. Mercuric oxide. The red variety was always used. If HgO is unavailable, lead sesquioxide (Pb_2O_3) may be used.

8. Salit's nickel centrifuge ashing tubes, 3.5 inches in length and $\frac{3}{4}$ inch in diameter.[1] The ashing tubes must never be allowed to come in contact with acids or strong alkalies. Immediately after use they are cleaned with brush and water; then the inside, especially that of the lower part, is rubbed with fine emery applied by a cloth wrapped around an iron rod. After thorough rinsing, they are washed with soap and water and then rinsed with tap water, distilled water, and absolute alcohol. When they are thoroughly dry, they are tightly stoppered with cork stoppers and kept in a dry place until further use.

9. Ordinary 15 cc. conical glass tubes, preferably with a slightly roughened inside surface near the tip to hold the precipitate in place when inverted.

10. Stirring rods. No. 1, 7 inches in length and 1.0 mm. in diameter. No. 2, 7 inches in length and 2 mm. in diameter. No. 3, 7 inches in length and 3 mm. in diameter. The first two are made by softening a glass tubing in the flame and drawing it out until the requisite fineness is obtained. The glass filament is then cut into rods and the ends are sealed in the flame.

11. A muffle furnace with thermostat.[2] The maximum temperature that can be used with this particular thermostat is 537.8°. If the ordinary muffle furnace, with rheostat, is used, one must watch the temperature and keep it below 480°.

Test experiments with this method were carried out on whole blood, serum, urine, and feces.

[1] Manufactured by the Precision Scientific Company, 1750 North Springfield Avenue, Chicago; approximate cost, $2.50 each.

[2] The muffle furnace used in these experiments was made by Dr. Norman K. Ceaglaske, Professor of Metallurgy, Department of Chemistry, State University of Iowa, Iowa City. It costs considerably less than the usual muffle furnaces with rheostat.

Technique for Whole Blood—Samples of 2 cc. of a 1:5 dilution, equivalent to 0.4 cc. of whole blood, are pipetted into nickel centrifuge ashing tubes. To each tube are added 2 cc. of a 3 per cent potassium-free gelatin solution and 2 cc. of absolute alcohol.[3] The mixture is evaporated to dryness in an electric drying oven at about 85°. That portion of the inside wall of the ashing tube covered by the residue is moistened with a few drops of absolute alcohol. Over this some mercuric oxide, equivalent in amount to a large wheat kernel or a small pea, is sprinkled from the point of a narrow knife blade or letter opener while the tube is held at an appropriate angle. The tubes are placed horizontally in the muffle furnace and the contents are allowed to ash overnight or some 10 or 12 hours at a temperature of 465°. After cooling, the part covered by the ash is again moistened with absolute alcohol and covered with a small amount of mercuric oxide as before. Ashing is continued for another 10 or 12 hour period. After cooling, 8 cc. of cold distilled water are pipetted into each ashing tube. The contents are stirred with a No. 3 stirring rod with frequent rubbing of the sides for a few minutes. The tubes are stoppered and centrifuged for 10 minutes at a speed of 4000 R.P.M. The supernatant fluid is decanted into a 15 cc. centrifuge tube, and an aliquot of 5 cc. is pipetted into another 15 cc. centrifuge tube. This contains an equivalent of 0.25 cc. of whole blood. (If duplicates are run or other additional analyses, such as for sodium, are made on the same sample, 1 cc. of whole blood is pipetted into the nickel centrifuge ashing tube. This is followed by the addition of 5 cc. of absolute alcohol, but the gelatin is omitted. After the contents are evaporated to dryness, the ashing is carried out as described in the first case. The ash is dissolved in 10 cc. of distilled water, the tube is centrifuged, and 2 cc., equivalent to 0.2 cc. of whole blood, are pipetted into a 15 cc. centrifuge tube.) At this point the following procedure is applicable in both cases. The contents of the centrifuge tubes are evaporated in an electric drying oven. At the same time, 4 cc. of a potassium standard solution, containing 0.4 mg. of K, in another 15 cc. centrifuge tube are similarly

[3] Alcohol is added to distribute the contents of the ashing tubes over a larger area as well as to impart sponginess to the residue, both states being conducive to a more thorough ashing. This applies also to such materials as the aqueous and vitreous humors, spinal fluid, and ascitic fluid.

treated. To both the samples and the standard is added 0.2 cc. of a 10 per cent chloroplatinic acid solution. By means of fine glass rods (No. 1) the chloroplatinic acid is thoroughly mixed with the residue. The tubes, with the stirring rods remaining inside, are allowed to cool in the ice box for 5 minutes. By means of a pipette, 5 cc. of ice-cold absolute alcohol are blown into the tubes, and the contents are vigorously stirred with stirring rods. After the rods are rinsed with a few drops of absolute alcohol, the tubes are stoppered with cork stoppers and allowed to stand in ice-cold water in the ice box for 20 minutes. Meanwhile the brass centrifuge cups are also cooled in the ice box and filled three-fourths full with ice-cold water. The tubes are centrifuged for 10 minutes at a speed of 4000 R.P.M. The supernatant alcohol is poured off and the tubes are placed in an inverted position on a piece of filter paper for a few seconds. The mouths of the tubes are wiped with filter paper, and the precipitate, with the help of a No. 2 stirring rod, is washed with 5 cc. of ice-cold absolute alcohol. The tubes are stoppered and centrifuged in precooled brass centrifuge cups as before. The supernatant alcohol is poured off and the tubes are placed in an inverted position on filter paper for a few seconds. After the mouths of the tubes have been wiped with filter paper, the tubes are placed horizontally in an electric drying oven and the precipitate is dried for a few minutes at a temperature of 65°. The precipitate is dissolved in about 5 cc. of distilled water, stirred constantly with a No. 3 stirring rod. The tubes are filled to within 1 inch from the top with distilled water from a wash bottle and stirring is continued for a few moments. The solution is transferred to a 100 cc. volumetric flask through a small long stem funnel (reaching below the neck of the flask). The tube is rinsed thoroughly several times and the washings are transferred to the volumetric flask. Distilled water is added to make the volume approximately 90 cc. To the flask are added 4 cc. of 1 N HCl (or H_2SO_4), and the contents are mixed. This is followed by the addition of 4 cc. of 72 per cent potassium iodide solution. Distilled water is added to the mark, and the flasks are well shaken. They are allowed to stand for half an hour in a dark place, after which they are again shaken. Shaking is repeated after another half hour's standing. The solutions are then ready for colorimetric readings. If the reading of the standard of 0.4 mg. of K is

20.0 and that of the sample of 0.25 cc. of whole blood is 17.8, the calculation is as follows:

$$\frac{20.0 \times 0.4 \times 100}{17.8 \times 0.25} = 179.8 \text{ mg. } \% \text{ K}$$

Recovery Tests—In these tests the samples were made up of standard potassium solution and blood in such proportions that each of the components represented approximately half of the total potassium content. The mixture was pipetted into the nickel centrifuge ashing tubes, evaporated, ashed, and carried through the rest of the process exactly as outlined for the pure blood sample. The results are listed in Table I.

Technique for Animal Serum—Since, as a rule, animal serum is available in sufficient quantities, somewhat greater accuracy is obtained by using 2 cc. instead of 1 cc. After the serum is pipetted into the ashing tubes, 3 cc. of absolute alcohol are added to each tube. The mixture is evaporated to dryness. The residue is ashed in the manner described for whole blood, and the ash is dissolved in 6 cc. of distilled water. After centrifuging, the supernatant solutions are poured off into small test-tubes. An aliquot of 5 cc., equivalent to 1.6667 cc. of original serum, is used for the analysis, as described for whole blood.

Recovery Tests—The samples for ashing were made up partly of standard potassium solution and partly of serum in the same way as those for whole blood. The results are given in Table II.

Technique for Human Serum—Since one has to be more economical with human serum than with animal serum, only 1 cc. of the former is used.[4] To the serum in the ashing tubes are added 5 cc. of absolute alcohol, and the mixture is evaporated to dryness. The residue is ashed as described for whole blood. The ash is dissolved in 6 cc. of distilled water, the tubes are centrifuged, and the supernatant solution is poured off into small test-tubes. An aliquot of 5 cc., equivalent to 0.8333 cc. of the original serum, is prepared for the analysis, as described for whole blood. A potassium standard containing only 0.3 mg. of K is used. The potassium chloroplatinate precipitates of both the standard and the samples are transferred to 50 cc. volumetric flasks by means

[4] If possible, 1.5 cc. should be used. The ash is dissolved in 6 cc. of distilled water. An aliquot of 5 cc. of this solution is equivalent to 1.25 cc. of the original serum.

of three washings. In order to leave space for the subsequent additions of 2 cc. of 1 N HCl and 2 cc. of 72 per cent KI solution,

TABLE I

Recovery Tests on Human Whole Blood

Subject No.	Experiment No.	K in blood	Recovery tests, blood + K standard		
			K calculated	K found	Per cent recovery
		mg. per cent	*mg.*	*mg.*	
I	1	175.2	0.3469	0.3448	99.4
	2	174.4	0.3460	0.3448	99.7
	3	171.1	0.3424	0.3429	100.1
	4	173.6	0.3452	0.3429	99.3
	5	170.6	0.3418	0.3429	100.3
	6	175.4	0.3354	0.3315	98.8
	7	175.1	0.3801	0.3846	101.2
	8	171.3	0.3770	0.3797	100.7
	9	170.5	0.3763	0.3750	99.7
II	1	192.2	0.2741	0.2760	100.7
	2	191.3	0.2731	0.2760	101.1
	3	191.3	0.2731	0.2727	99.9
	4	190.0	0.2720	0.2727	100.3
	5	190.0	0.2720	0.2727	100.3
	6	187.5	0.2700	0.2727	101.0
	7	191.3			
	8	190.0			
	9	191.3			
	10	191.3			
	11	191.3			

TABLE II

Recovery Tests on Ox Serum

Animal No.	K in serum	Recovery tests, serum + K standard		
		K calculated	K found	Per cent recovery
	mg. per cent	*mg.*	*mg.*	
I	31.0	0.4080	0.4081	100.0
II	24.2	0.3536	0.3550	100.4
III	42.1	0.4964	0.4918	99.1
IV	25.7	0.3654	0.3681	100.7

the total amount of washings should not exceed 45 cc. The rest of the procedure is as described for whole blood. The results are

somewhat less accurate than those on 2 cc. samples, but, on the whole, quite satisfactory, as is seen from the deviations of the mean of twenty-one determinations (Table III).

Technique for Urine—First a 1:20 dilution of urine is prepared by diluting 5 cc. of urine to 100 cc. Exactly 5 cc. of this diluted urine, equivalent to 0.25 cc. of the original urine, are pipetted into ashing tubes. To the samples are added 2 cc. of 3 per cent gelatin solution, and the mixture is evaporated to dryness. Ashing of the residue is carried out exactly as described for whole blood. The ash is dissolved in 10 cc. of distilled water, and the tubes are centrifuged. The supernatant fluid is poured off into a test-tube, and an aliquot of 5 cc., equivalent to 0.125 cc. of the original urine, is evaporated in a 15 cc. centrifuge tube. The rest

TABLE III

Deviations of Mean with 1 Cc. Serum Samples

Experiment No.	Colorimeter reading	Mean deviation	Experiment No.	Colorimeter reading	Mean deviation	Experiment No.	Colorimeter reading	Mean deviation
1	22.4	0	8	22.4	0	15	22.8	+1.2
2	22.3	−0.6	9	22.6	+0.8	16	21.7	−3.2
3	22.4	0	10	23.1	+3.0	17	21.8	−2.8
4	21.9	−2.4	11	22.3	−0.6	18	22.7	+1.2
5	22.3	−0.6	12	22.4	0	19	22.4	0
6	23.1	+3.0	13	22.4	0	20	22.5	+0.3
7	22.6	+0.8	14	22.5	+0.3	21	22.4	0

of the procedure is the same as that described for whole blood, including a standard of 0.4 mg. of K and 100 cc. volumetric flasks for the final solution in which color is developed.

Recovery Tests—The samples for ashing were made up partly of standard potassium solution and partly of urine in a similar way to those in the recovery tests for whole blood. The results are given in Table IV.

Technique for Feces—Exactly 5 gm. of feces are transferred to a 100 cc. lipless glass cylinder. Distilled water is added to the 100 cc. mark, and the cylinder is stoppered with a rubber stopper. The contents are well shaken and the cylinder is allowed to stand overnight at room temperature. After several more shakings, 5 cc. samples of the emulsion are measured out into the nickel centrifuge

ashing tubes by means of a calibrated glass tubing, $\frac{1}{4}$ inch in diameter and with the tip slightly constricted. This quantity of

TABLE IV

Recovery Tests on Urine

Specimen No.	Experiment No.	K in urine	Recovery tests, urine + K standard		
			K calculated	K found	Per cent recovery
		mg. per cent	*mg.*	*mg.*	
I	1	383.2	0.3685	0.3640	98.9
	2	383.2	0.3685	0.3660	99.4
	3	384.6	0.5513	0.5464	99.1
	4	384.6	0.5513	0.5406	98.1
II	1	400.0	0.5000	0.5000	100.0
	2	400.0	0.5000	0.5031	100.6
III	1	398.0	0.4983	0.4969	99.7
	2	398.0	0.4983	0.4969	99.7
IV	1	480.0	0.5667	0.5674	100.1
	2	472.0	0.5605	0.5661	101.0
V	1	329.0	0.3974	0.4054	102.0
	2	329.0	0.3974	0.3974	100.0
	3	329.0	0.3974	0.3974	100.0
	4	329.0	0.3179	0.3062	96.3
	5	329.0	0.3179	0.3191	100.4
	6	329.0	0.3179	0.3169	99.7
VI	1	367.5	0.2188	0.2184	99.8
	2	367.5	0.2183	0.2184	100.1

TABLE V

Deviations of Mean in Experiments with Feces

Experiment No.	K	Mean deviation
	mg. per cent	*per cent*
1	369.9	−0.2
2	372.1	+0.4
3	369.9	−0.2
4	371.1	+0.1
5	369.9	−0.2

emulsion is equivalent to 0.25 gm. of the original feces. To the emulsion in the ashing tubes are added 2 cc. of a 3 per cent gelatin solution, and the mixture is evaporated to dryness. The residue

is ashed exactly as described for whole blood. The ash is dissolved in 10 cc. of distilled water. After centrifuging, an aliquot of 5 cc., equivalent to 0.125 gm. of the original feces, is evaporated in a 15 cc. centrifuge tube. The rest of the procedure is exactly as described for whole blood.

No recovery tests were made on feces, but as is seen from the deviations of the mean of five feces samples taken from the contents of the same cylinder, the results are very satisfactory (Table V).

SUMMARY

A microcolorimetric method for potassium determinations in biological materials is described. The material is ashed in specially made nickel centrifuge ashing tubes in the presence of HgO at a temperature of 465°. The potassium in the soluble ash is determined by an application of Shohl and Bennett's colorimetric chloroplatinate method.

Details are given for whole blood, serum, urine, and feces.

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ESTIMATION OF "ACID" PHOSPHATASE ACTIVITY OF BLOOD SERUM

By ETHEL BENEDICT GUTMAN AND ALEXANDER B. GUTMAN

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)

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To distinguish them from the better known "alkaline" phosphatases, phosphomonoesterases with optima on the acid side of neutrality are referred to as "acid" phosphatases (1). The quantitative determination of these enzymes in serum is of interest chiefly because (*a*) the acid phosphatase activity of normal blood serum (2–4) is attributed to distinct phosphomonoesterases of uncertain physiological significance (5), and (*b*) the blood serum of subjects with metastasizing carcinoma of the prostate gland contains an acid phosphatase (6) with properties corresponding to the enzyme found by Kutscher and Wolbergs (7) in normal prostate tissue and in seminal fluid. Invasion of the circulating fluids by carcinomatous prostate tissue liberates prostate acid phosphatase into the blood where the enzyme can be identified and the source of the primary tumor so determined.

The general principles underlying the determination of serum "alkaline" phosphatases apply also to the determination of serum "acid" phosphatases. We have adapted the King and Armstrong method for alkaline phosphatase (8) to the estimation of serum acid phosphatase activity (5, 6). Barringer and Woodard (9) have suggested a variant of the Bodansky method (10); Lundsteen and Vermehren (11) have modified their own method (12) for the same purpose. We wish to consider here certain specific conditions which must be satisfied in adaptations of this kind: (*a*) Since serum contains both alkaline and acid phosphatases, the former usually in great excess, hydrolysis must be conducted under conditions which are optimal for acid but completely inhibit alkaline enzymes. This involves a study of pH-activity relations,

particularly of pathological sera containing varying proportions of both enzymes. (*b*) The acid phosphatase activity of normal sera is extremely small. A number of substrate-buffer combinations in varying concentrations were investigated in order to obtain satisfactory colorimetric readings without having to hydrolyze too long. After prolonged hydrolysis significant deviations from linear time-activity relations occur.

Selection of pH—Fig. 1 illustrates the pH-activity relations of the serum acid phosphatases of principal interest, that normally

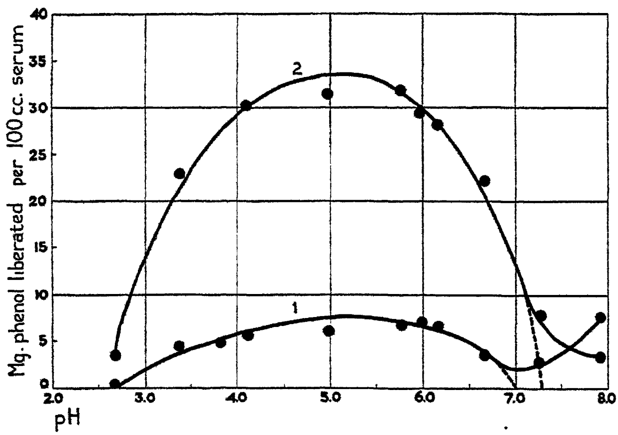

Fig. 1. pH-activity curves (0.005 M monophenyl phosphate substrate, 0.2 M acetate buffer, t = 37°). Curve 1, normal human serum, 3 hours hydrolysis; Curve 2, subject with metastasizing prostatic carcinoma, serum diluted 1:20, ½ hour hydrolysis.

present (Curve 1) and that occurring in the serum of patients with metastasizing prostatic carcinoma (Curve 2). Under the stated conditions of hydrolysis, there is a rapid decline in activity outside of the approximate limits of pH 4.0 to 6.0, the pH range suitable for estimating serum acid phosphatases as defined by optimal zones of activity.

The curves in Fig. 1 are discontinuous in the region of neutrality, where alkaline phosphatase activity, though only a fraction of that at the pH optimum, is nevertheless sufficient to be significant in relation to the acid phosphatase activity of most sera. In experiments such as those summarized in Table I, we have at-

tempted to determine how far on the acid side of neutrality inactivation of serum alkaline phosphatase is incomplete, particularly in sera extremely rich in alkaline phosphatase (Paget's disease). The hydrolyses were conducted in the presence of 0.02 M NaF, which inhibited serum acid phosphatase to minimal activity (0.1 to 0.2 unit at pH 5.0). The results with monophenyl phosphate substrate (Table I) show measurable activity of serum alkaline phosphatase as far as pH 6.0. Similar experiments with β-glycerophosphate substrate reveal measurable alkaline phosphatase activity at least as far as pH 6.0.[1] Barringer and Woodard (9),

TABLE I

Activity of Serum "Alkaline" Phosphatase at Decreasing pH Levels; Serum "Acid" Phosphatase Inhibited by Fluoride

0.005 M monophenyl phosphate substrate and 0.2 M acetate buffer were used in the presence of 0.02 M NaF; $t = 37°$. The figures show the mg. of phenol liberated per hour per 100 cc. of serum.

Serum No.	pH*								
	9.4	8.8	8.28	7.92	7.27	6.68	6.17	5.96	4.97
1	64.2	19.2	15.9	14.3	3.1	1.0	0.7	0.7	0.2
2	434	176	144	108	14.5	3.3	1.3	1.1	0.1
3	10.4		2.6	2.5	0.9	0.6	0.6	0.5	0.2

* pH of substrate-buffer-serum reaction mixture as determined by glass electrode-vacuum tube potentiometer at 25°. At pH 9.4, veronal buffer was used.

using β-glycerophosphate substrate, likewise noted appreciable serum alkaline phosphatase activity at pH 6.4.

In view of the results indicated, we have elected to buffer at pH 4.9, which is within the zone of optimum activity of serum acid phosphatases and beyond the range of significant activity of serum alkaline phosphatases under the conditions of hydrolysis specified.

[1] The substrate used affects the results of such experiments in two ways: As pointed out by King and Delory (13), and in accord with the Michaelis-Menten equation, the optimum pH for hydrolysis of phosphoric esters by alkaline phosphatase increases with increasing acidity of the ester; and the rate of decline in activity exhibited by alkaline phosphatase when buffered at increasingly acid levels decreases with increasing acidity of the ester.

Selection of Substrate—Within the same period of time, 2 to 3 times as much phenol is split off from monophenyl phosphate by serum acid phosphatase at pH 5.0 as phosphate (expressed as inorganic phosphorus) from β-glycerophosphate (Table II).[2] With α-glycerophosphate substrate, the proportion in favor of monophenyl phosphate is even greater (5, 6, 11). Because of the low range of activity of most sera and the theoretical and practical advantages of short periods of hydrolysis, monophenyl phosphate would seem, therefore, to be particularly suitable for the estimation of serum acid phosphatases. Phenol can be quantitatively estimated in the hydrolysate with an accuracy at least as great as is possible with phosphate.

Table II

Comparison of β-Glycerophosphate with Monophenyl Phosphate As Substrate for Serum "Acid" Phosphatases; Comparison of Acetate and Citrate Buffers at pH 5.0

The values are given in mg. of P or phenol per hour per 100 cc. of serum.

Serum No.	M/60 β-glycerophosphate substrate, 0.2 M acetic acid-acetate buffer (a)	0.005 M monophenyl phosphate substrate: 0.2 M acetic acid-acetate buffer (b)	0.005 M monophenyl phosphate substrate: 0.1 M citrate-NaOH buffer (c)	(b)/(a)	(b)/(c)
	mg. P	*mg. phenol*	*mg. phenol*		
1*	525	1052	985	2.0	1.07
2*	100	270	261	2.7	1.03
3*	80	204	193	2.5	1.06
4*	8.6	18.1	15.8	2.1	1.15
5*	1.6	3.3	3.1	2.1	1.06
6	1.1	3.0	1.9	2.7	1.58
7	0.7	2.0	1.6	2.9	1.25
8	0.8	2.0	1.4	2.5	1.43

* Obtained from subjects with metastasizing prostatic carcinoma.

The concentration of phosphoric ester substrate in the hydroly-

[2] This ratio of about 2.5:1 is considerably less than that observed with phosphatases in alkaline medium, because at pH 5.0 the rate of hydrolysis of monophenyl phosphate is slower than that of β-glycerophosphate (*cf.* (13)).

sis mixture is another factor determining the rate of scission, which increases with increasing molarity of the substrate. With β-glycerophosphate, comparatively small increases in substrate concentration effect large differences in the amount of phosphate liberated. The rate of hydrolysis of monophenyl phosphate in acid medium, however, is much less susceptible to changes in substrate concentration; between 0.005 M and 0.01 M the differences in phenol split off are small (6).

Impurities in the disodium monophenyl phosphate employed as substrate affect the rate of liberation of phenol by alkaline (14, 15) and acid phosphatases. With sera of normal or moderately increased acid phosphatase activity, the use of pure disodium monophenyl phosphate (for which we are indebted to Mr. H. Scharer) gave values up to 15 per cent higher than those obtained with commercial products. In sera exhibiting marked acid phosphatase activity, however, the increase did not exceed 4 per cent.

Selection of Buffer—In estimating serum acid phosphatases with monophenyl phosphate substrate, it was found that acetate-acetic acid, citrate-NaOH, succinic acid-borax, veronal-acetic acid, or glycine-HCl could be employed to buffer at pH 4.9. With β-glycerophosphate substrate, a variety of buffers could also be used but difficulties arise, as for example with citrate buffer; in 0.1 M concentration, the development of color in determining liberated phosphate with molybdic acid is inhibited (16) (0.04 M citrate, however, does not inhibit color development and is an adequate buffer (5)).

With monophenyl phosphate substrate, acetate-acetic acid buffer (suggested by Lundsteen (16)) consistently gave higher values for serum acid phosphatase activity than did citrate (Table II) and was our buffer of choice for many purposes. In subjects with metastasizing prostatic carcinoma the differences in values obtained with these two buffers was small ((*b*)/(*c*), Table II), whereas in patients with other bone conditions and in normal subjects the differences were large; *i.e.*, 0.1 M citrate appreciably inhibits the activity of serum acid phosphatases other than the prostate acid phosphatase which appears in the serum in association with metastasizing prostatic carcinoma. This observation is of interest in two connections: In doubtful cases, it permits the differentiation of prostate acid phosphatase from other acid phos-

phatases in the serum; and by means of citrate buffer it is possible to establish a sharper critical level for diagnostic purposes (3.0 units of acid phosphatase activity per 100 cc. of serum). For example, with acetate buffer the serum of a patient with Paget's disease (Serum 6, Table II) gave a value of 3.0 units, within the range of values obtained with metastasizing prostatic carcinoma, but with citrate buffer gave a value of only 1.9 units. The serum of a patient with metastasizing prostatic carcinoma (Serum 5, Table II) gave a value of 3.3 units with acetate buffer, 3.1 units with citrate buffer.

Selection of Method—It appeared from the foregoing experiments that the principles of the King and Armstrong method for determining alkaline phosphatase activity (8) could be applied advantageously to the estimation of serum acid phosphatases. The results of recent critical studies and suggested variants of the King and Armstrong method (17–19, 15) were taken into account in our adaptation.

Reagents—

1. Buffer-substrate (0.005 M monophenyl phosphate, 0.1 M citrate at pH 4.9). Mix equal parts of Solutions A and B as needed; check pH.

Solution A. Dissolve 1.09 gm. of disodium phenyl phosphate (obtainable from Eimer and Amend, New York) in 500 cc. of water.

Solution B. Dissolve 42.0 gm. of crystalline citric acid in water, add 376 cc. of 1 N NaOH, make up to 1 liter. Adjust the pH to 4.9 with NaOH or HCl as needed (when colorimetric methods are used, nitrazine (Squibb) is a convenient indicator). Preserve in well stoppered bottles in a refrigerator.

2. Phenol reagent of Folin and Ciocalteu (20). The stock reagent is prepared as described by Folin and Ciocalteu, kept in a well stoppered, amber bottle, and for use diluted 1:3.

3. Sodium carbonate (20 per cent solution).

4. Standard phenol. For the stock solution, which keeps indefinitely, dissolve 1 gm. of crystalline phenol in 0.1 N HCl and make up to 1 liter with 0.1 N HCl. Standardize by the convenient method of Koppeschaar (21). From this stock phenol solution, a diluted phenol solution containing *exactly* 10 mg. of phenol per 100 cc. is made up; it remains stable for months in the refrigerator.

5. Standard phenol solution and reagent. To 1 cc. of diluted phenol solution in a test-tube, add 6 cc. of distilled water and 3 cc. of diluted phenol reagent. Prepare shortly before use.

Procedure

Two test-tubes, each containing 10 cc. of buffer-substrate solution, are kept in a water bath at 37° for about 5 minutes. Pipette exactly 0.5 cc. of the serum to be tested into each tube, stopper, mix, and incubate at 37° for 3 hours. Then remove the tubes from the water bath, at once add 4.5 cc. of diluted phenol reagent, mix, and filter. To two control tubes each containing 10 cc. of buffer-substrate solution add 0.5 cc. of the serum and at once add 4.5 cc. of diluted phenol reagent, mix, and filter.

Pipette 6 cc. of each of the test and control filtrates into test-tubes, add 1.5 cc. of 20 per cent sodium carbonate solution, and mix. To the standard phenol solution and phenol reagent (No. 5) prepared shortly before, add 2.5 cc. of 20 per cent sodium carbonate solution and mix. Place test, control, and standard tubes together in the water bath for 5 minutes to develop the color, cool about 20 minutes, and compare in the colorimeter.[3] (Although the color is not maximally developed, the standard, test, and control tubes are all made up at the same time and read within a few minutes of each other.) The unknown is placed in the left cup of the colorimeter and set at 30. The standard is placed in the right cup, which is adjusted to match the unknown.

Calculation

The results are expressed in units of acid phosphatase activity per 100 cc. of serum. A unit is defined as that degree of acid phosphatase activity which at 37° will liberate from the specified buffer-substrate solution (pH 4.9) 1 mg. of phenol in 1 hour. The units of phosphatase activity in 100 cc. of serum = mg. of phenol present in 100 cc. of serum after hydrolysis *minus* mg. of "phenol" in 100 cc. of the non-incubated control serum *divided by* the number

[3] The Evelyn photoelectric colorimeter can be used advantageously. Proceed as indicated, except allow the color to develop 1 hour for stabilization. The readings are approximately 10 per cent higher than with the ordinary colorimeter.

of hours of hydrolysis. To calculate the mg. of phenol in 100 cc. of serum before and after hydrolysis,

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{strength of standard} \times$$

$$\frac{\text{final volume of unknown}}{\text{final volume of standard}} \times \frac{100}{\text{cc. serum used}}$$

With the unknown set at 30, the standard containing 0.1 mg. of phenol and the reagents used in the proportions indicated, this equation becomes

$$\frac{\text{Reading of standard}}{30} \times 0.1 \times \frac{7.5}{12.5} \times \frac{15}{6 \times 0.5} \times 100$$

which cancels out to "reading of standard." The final result, units of acid phosphatase activity per 100 cc. of serum, is obtained as follows: reading of standard against incubated serum *minus* reading of standard against control serum *divided by* the number of hours of hydrolysis.

With sera of increased acid phosphatase activity it may be more convenient to set the unknown at 20, 15, or 10, and use the appropriate factors 3/2, 2, or 3. In the case of sera with very high acid phosphatase activity (when the reading of standard is over 60), there is marked inhibition of hydrolysis by the products of scission and to obtain optimal values it is necessary to reduce the time of hydrolysis. When $\frac{1}{4}$ hour's hydrolysis still gives too high readings, the serum must be diluted appropriately with physiological saline solution and the determination repeated, including controls with the diluted serum. Over periods of hydrolysis from $\frac{1}{4}$ to 3 hours, time-activity curves are linear except with sera very high in acid phosphatase activity, and these are linear if diluted as indicated. Significant deviations from Beer's law were not observed.

Results

In the past 2 years, we have employed the method described in over 500 determinations of serum acid phosphatase activity in a variety of conditions. Agreement between duplicates is usually within 0.2 unit in the normal range and better than 5 per cent in sera with elevated values. The normal range was found to be

0.5 to 2.0 unit per 100 cc. of serum, with increases to several hundredfold in subjects with metastasizing prostatic carcinoma (22, 23).

SUMMARY

Optimal conditions of hydrolysis in the estimation of serum "acid" phosphatases were determined. The King and Armstrong method for "alkaline" phosphatases was adapted to the estimation of serum "acid" phosphatases.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LXI. THE POLYSACCHARIDE OF THE PHOSPHATIDE OBTAINED FROM CELL RESIDUES IN THE PREPARATION OF TUBERCULIN*

By R. J. ANDERSON, ROBERT L. PECK,† and M. M. CREIGHTON†

(*From the Department of Chemistry, Yale University, New Haven*)

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It has been shown in earlier investigations (1) that the phosphatide of the human tubercle bacillus is easily saponified when treated with dilute alkali at room temperature and that the cleavage products consist of about 64 per cent of fatty acids and about 40 per cent of water-soluble substances. The compounds soluble in water consisted of inorganic and organic phosphoric acids and a phosphorus-containing polysaccharide. The polysaccharide could be dephosphorylated by heating with dilute ammonia in a sealed tube. The phosphorus-free polysaccharide, on hydrolysis with dilute acid, gave only mannose and inosite in the ratio of 2:1. The polysaccharide was therefore regarded as a new glycoside which was named manninositose, but, since it did not crystallize, its purity could not be established.

The cleavage products of the polysaccharide mentioned above were different from those obtained when the phosphatide was hydrolyzed directly with dilute aqueous acid (2). In the latter case the fatty acids were split off and the polysaccharide component was hydrolyzed completely, yielding inorganic and organic phosphoric acids, together with inosite, mannose, and some other reducing sugar which gave glucosazone on treatment with phenylhydrazine.

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† Holder of a National Tuberculosis Association Fellowship in Chemistry at Yale University, 1939-40.

In view of the difference in the simple carbohydrates produced from the polysaccharide on acid hydrolysis and after alkaline saponification, it appeared of interest to study the composition of manninositose more closely, but to do so would require larger quantities of phosphatide than were available. That used in previous investigations had been prepared from living tubercle bacilli especially provided for our work by the H. K. Mulford Company and by Sharp and Dohme, but at such high cost that a cheaper source was desired. The bacillary residues which are a by-product in tuberculin manufacture and in the production of the purified protein derivative, PPD, of Seibert and coworkers (3) yield a phosphatide which at first appeared to be identical in properties with the original product. It will be shown in the present report, however, that the polysaccharide which was isolated after the phosphatide had been saponified with alkali was not identical with the glycoside described under the name of manninositose.

Two phosphatide preparations were used that had been prepared from two separate lots of bacterial residues. The polysaccharides from each preparation on being heated to 170–180° with 14 per cent ammonia did not yield identical cleavage products and they also differed in the ease with which the phosphorus was split off. After dephosphorylation the first polysaccharide gave inorganic phosphate, inosite, and a glycoside which on hydrolysis gave approximately equal parts of mannose and some other reducing sugar from which glucosazone was obtained on treatment with phenylhydrazine. The second polysaccharide gave inorganic phosphate, inosite, and a glycoside which on hydrolysis yielded about equal parts of inosite, mannose, and some other reducing sugar. The glycoside manninositose (1) which we obtained in our earlier investigations after the polysaccharide had been dephosphorylated could not be found. The dissimilarity in cleavage products observed in the present investigations must depend upon differences in linkage of the carbohydrate molecules in the polysaccharides.

EXPERIMENTAL

The bacterial residues used in this investigation were derived from a strain of the human tubercle bacillus which was obtained from the Bureau of Animal Industry and designated by Sharp and Dohme as Strain 2067. Two separate lots of residues were ex-

tracted at different times. Both lots of bacilli had been grown on the Long (4) synthetic medium for periods of about 8 weeks, after which the cultures were heated to 100° for 3 hours and afterwards held in a refrigerator at 3–5° for about 1 week. The bacterial cells, after being filtered off and washed with a phosphate buffer solution, were heated in an autoclave to 120° for 30 minutes. We are indebted to Sharp and Dohme, Glenolden, Pennsylvania, for this material.

The extraction of the bacterial residues and the separation of the lipids into phosphatide, acetone-soluble fat, and wax were carried out as described in former papers (5).

Phosphatide I

The phosphatide from the tubercle bacilli residues was a nearly white granular powder which melted with decomposition at 204–205°; when rubbed up with water, it formed a colloidal solution. On analysis the following values were obtained: P 3.52, N 0.61, ash 12.02 per cent.

Saponification of Phosphatide I

For saponification 40.0 gm. of the phosphatide were dissolved in 400 cc. of benzene and to the solution were added 7.0 gm. of potassium hydroxide dissolved in 50 cc. of warm absolute alcohol. When the solutions were mixed, the color turned light orange and after a few minutes a gelatinous precipitate began to separate. After the mixture had stood at room temperature for 2 days, the solution had set to a stiff gel. When it was warmed slightly, the gel dissolved, leaving a finely divided precipitate. Since it was impossible to filter off the precipitate, the mixture was transferred to a separatory funnel, diluted with water, and acidified with acetic acid. After the mixture had been thoroughly shaken, the aqueous layer was drawn off and extracted twice with ether. The aqueous solution was examined for carbohydrate, etc., as will be described later.

Isolation of Fatty Acids

The ethereal extracts and the benzene solution obtained as above were combined and evaporated to dryness. The residue was refluxed for 2 hours with alcoholic potassium hydroxide, after which

the fatty acids were isolated in the usual manner and dried. The acids formed a slightly brownish solid at room temperature and weighed 26.6 gm., which is equal to 66.5 per cent of the phosphatide. The acids were investigated as will be described in a separate paper.

The Water-Soluble Compounds. Isolation of Phosphoric Acids—The ether-extracted aqueous solution was concentrated *in vacuo* to a volume of about 25 cc. and neutralized with ammonia, after which a solution of neutral lead acetate was added until no further precipitate separated. The lead salts were filtered off, washed with water, and decomposed in aqueous suspension with hydrogen sulfide. After the lead sulfide had been filtered off, the filtrate was concentrated *in vacuo* to a volume of about 20 cc. and neutralized with barium hydroxide. A white precipitate of barium phosphate separated, which was filtered off, washed with water, and dried *in vacuo*; it weighed 0.48 gm. The filtrate was diluted with 2 volumes of alcohol, whereupon a white flocculent precipitate appeared, which was collected, washed with alcohol, and dried *in vacuo*. This substance, which consisted of the barium salt of an organic phosphoric acid, weighed 0.36 gm. It was dissolved in water, filtered from a trace of insoluble matter, and reprecipitated by adding 2 volumes af alcohol. The snow-white amorphous powder weighed 0.27 gm., and gave a strongly positive Scherer reaction, indicating an inosite phosphoric acid. For analysis the substance was dried at 78° *in vacuo*.

$C_6H_{11}O_9PBa$ (395.4).	Calculated.	Ba 34.75,	P 7.84
	Found.	" 35.78, 35.79;	" 8.18, 8.27

Isolation of Polysaccharide

The polysaccharide contained in the filtrate from the lead salt mentioned above was precipitated by means of basic lead acetate and ammonia in the usual manner. The filtrate was examined for glycerol, as will be described later.

The lead precipitate was decomposed with hydrogen sulfide. The filtrate was concentrated *in vacuo* to a thick syrup and the latter was dehydrated by grinding in a mortar under absolute alcohol until a white powder was obtained. The product weighed 6.9 gm. The alcoholic solution on evaporation to dryness left a syrupy residue weighing 0.9 gm. which was not examined.

Examination for Glycerol—The filtrate from the basic lead acetate precipitate was concentrated *in vacuo* until the free ammonia was removed, after which the lead was removed with hydrogen sulfide. The filtrate was concentrated *in vacuo* to a syrup, mixed with an excess of barium hydroxide solution, and freed of previously combined ammonia by distillation under reduced pressure. The barium was then precipitated quantitatively with sulfuric acid, and the filtrate concentrated *in vacuo* to a syrup. The syrup gave no Molisch reaction, thus indicating absence of carbohydrate, but it contained much potassium acetate. It was dissolved in alcohol and concentrated hydrochloric acid was added in slight excess. Potassium chloride was filtered off and the filtrate was concentrated *in vacuo* to a thick syrup. The syrup was treated

TABLE I

Cleavage Products from 40 Gm. of Phosphatide I

	gm.
Fatty acids	26.6
Polysaccharide, white powder	6.9
" as syrupy residue	0.9
Glycerol	3.1
Inorganic barium phosphate	0.48
Barium salt of organic phosphoric acid	0.36
Total recovered	38.34

repeatedly with absolute alcohol in order to remove the remaining potassium chloride and evaporated to dryness *in vacuo*. The syrupy residue weighed 3.1 gm. and consisted of crude glycerol, identified by means of the tribenzoyl derivative which melted at 76°.

The cleavage products obtained from 40 gm. of the phosphatide are given in Table I. The apparent loss would be covered largely by the inorganic basic constituents of the ash in the phosphatide.

Dephosphorylation of Polysaccharide

The polysaccharide mentioned in a preceding paragraph was a white, amorphous, somewhat hygroscopic, powder which showed an acid reaction to litmus. It gave no reduction with Fehling's

solution until it had been boiled for several minutes with dilute sulfuric acid.

Analysis—Found, P 7.94, N 3.94

The phosphorus was split off when 5 gm. of the polysaccharide dissolved in 20 cc. of 14 per cent ammonia were heated in a sealed tube to 170° for $8\frac{3}{4}$ hours. The straw-colored solution was transferred to a distilling flask and concentrated *in vacuo* until the free ammonia was removed, after which a slight excess of barium hydroxide was added. The precipitate of barium phosphate was filtered off, washed with water, and dried. It weighed 3.9 gm.

The filtrate was concentrated *in vacuo* until the ammonia was removed, after which the excess of barium was removed quantitatively with sulfuric acid, and the filtrate was treated with norit. The colorless filtrate was concentrated to a thick syrup *in vacuo.* Very soon large, colorless, plate-shaped crystals began to separate. After complete crystallization the crystals were stirred up with cold 50 per cent alcohol, filtered off, and washed with 50 per cent alcohol, and with alcohol. The dried crystals weighed 1.07 gm.

The filtrate on evaporation to dryness left a glassy mass that weighed 2.5 gm. and was free from phosphorus and nitrogen. This material was treated as will be described later.

Identification of Inosite—The crystalline substance mentioned above after two recrystallizations from water by the addition of alcohol was obtained as colorless prismatic crystals that weighed 1.0 gm. The crystals gave the reaction of Scherer and melted at 225°. A mixed melting point with inactive inosite showed no depression. The substance was therefore pure inosite.

Acetylation of Non-Crystalline Material

The non-crystalline fraction, 2.5 gm., was acetylated in a mixture of pyridine and acetic anhydride. The reaction mixture was poured into dilute sulfuric acid and the acetyl derivative was extracted with chloroform. On evaporation of the solvent the resulting thick gum (4.4 gm.) was dissolved in about 10 cc. of methyl alcohol and very soon colorless prismatic crystals began to separate. The crystals were filtered off after the solution had stood in the refrigerator for 3 days, and washed with cold methyl alcohol. The filtrate and washings were concentrated to a thin syrup and

again cooled in the refrigerator, when another small crop of crystals was obtained. The two crops of crystals weighed 0.32 gm. and consisted of inosite hexaacetate, m.p. 211–212°. The reaction of Scherer was positive.

The filtrate on evaporation to dryness left a colorless, glassy mass which weighed 3.9 gm. The substance was extremely soluble in the usual organic solvents except petroleum ether and could not be crystallized from any solvent. A concentrated solution in methyl alcohol when mixed with cold water gave only a milky emulsion from which no solid particles separated.

The acetyl value was determined.

Analysis—Found, CH_3CO 56.1

Saponification of the Acetyl Derivative—Since it was impossible to crystallize the acetyl derivative, it was saponified by refluxing in methyl alcoholic solution with aqueous barium hydroxide. The free carbohydrate was isolated as a colorless glassy mass which weighed 1.9 gm. All attempts to crystallize it were unsuccessful but a solid fraction was finally obtained as follows: The substance was dissolved in 4 cc. of water and 5 cc. of alcohol were added. The faintly cloudy solution was poured into 150 cc. of absolute alcohol, whereupon a fine amorphous precipitate separated. The precipitate was collected, washed with absolute alcohol, and dried *in vacuo*. The snow-white powder, designated Fraction I, weighed 0.92 gm.

The alcoholic solution on evaporation to dryness left a sticky, gum-like residue which weighed about 1 gm. This material was designated Fraction II.

Examination of Carbohydrate Fraction I

The substance had no melting point. Heated in a capillary tube, it began to swell at 150–160° and there was slight effervescence at 217–220°. The Scherer reaction was negative; hence inosite was absent. It gave no reduction with Fehling's solution. When it was refluxed with 5 per cent sulfuric acid, the maximum reduction was obtained in 2.5 hours and amounted to 56 per cent, calculated as mannose.

Rotation—0.1705 gm. of substance dissolved in water and diluted to 10 cc. gave in a 1 dm. tube $\alpha = +1.26°$; hence $[\alpha]_D^{20} =$

+73.9°. There was no mutarotation. The substance was very soluble in water and insoluble in alcohol and it could not be crystallized.

Hydrolysis of Fraction I. Isolation of Mannose Phenylhydrazone—For the hydrolysis 0.2234 gm. of Fraction I was dissolved in 20 cc. of 5 per cent sulfuric acid and the solution was refluxed for 4 hours. After the sulfuric acid had been removed quantitatively with barium hydroxide, the solution was concentrated *in vacuo* to 8 cc. and mixed with 0.3 gm. of phenylhydrazine dissolved in a few drops of alcohol. The crystalline hydrazone which separated weighed 0.1741 gm. and after recrystallization from 60 per cent alcohol melted with decomposition at 195–196°; there was no depression of the melting point on admixture with pure mannose phenylhydrazone.

If the carbohydrate had been hydrolyzed only into mannose, the yield of mannose phenylhydrazone should have been 0.3526 gm., but the actual yield of hydrazone was only about one-half of this amount. Accordingly, some other sugar must have been formed also.

The excess of phenylhydrazine was removed with benzaldehyde, after which the solution was thoroughly extracted with chloroform and concentrated *in vacuo* to a thick syrup which could not be crystallized. This was insoluble in absolute alcohol, neutral to litmus, and strongly reduced Fehling's solution. The reaction of Scherer was negative, thus showing that inosite was absent.

To the syrup in 2 cc. of water 0.3 gm. of phenylhydrazine hydrochloride and 0.4 gm. of sodium acetate were added and the mixture was warmed until the reagents had dissolved. No hydrazone separated, thus indicating that mannose had been removed completely. When the solution was heated in a boiling water bath, a small amount of an osazone separated, which, after recrystallization from 50 per cent alcohol, melted with decomposition at 208°. The melting point corresponds to that of glucosazone.

The results obtained indicate that the carbohydrate of Fraction I gave about equal parts of mannose and some other reducing sugar on hydrolysis.

Carbohydrate Fraction II

The substance after it had been dried to constant weight at 78° *in vacuo* formed a transparent glassy mass.

Rotation—0.1013 gm. of substance dissolved in water and diluted to 10 cc. gave in a 1 dm. tube $\alpha = +0.43°$; hence $[\alpha]_D^{20} = +42.4°$.

For hydrolysis 0.8780 gm. of the carbohydrate was refluxed for 3.5 hours in 25 cc. of 5 per cent sulfuric acid and the reaction mixture was worked up as described for Fraction I. In this case the yield of mannose phenylhydrazone, m.p. 195–196°, was only 0.2 gm. The filtrate from the hydrazone gave 0.12 gm. of glucosazone, which melted with decomposition at 208°.

It is evident from the results obtained that the carbohydrate fractions differed in composition. Both fractions when hydrolyzed gave mannose and some other reducing sugar, but in very different amounts. The mannose was easily identified as the phenylhydrazone, but the nature of the mixture of other sugars remains unknown except for its ability to yield glucosazone.

TABLE II

Cleavage Products from 40 Gm. of Phosphatide II

	gm.
Fatty acids	26.9
Crude polysaccharide as syrup	9.2
" glycerol	1.95
Inorganic barium phosphate	0.30
Barium salt of organic phosphoric acid	0.27
Total recovered	38.62

Phosphatide II

In order to check the results reported above a new lot of polysaccharide was prepared from Phosphatide II which had been obtained from a separate lot of bacterial residues of Strain 2067.

The saponification of 40 gm. of the phosphatide, which appeared to be identical in properties with Phosphatide I, was carried out exactly as described before. In this experiment the gelatinous precipitate that separated could be filtered off and was washed with benzene and with ether. In order to remove adhering fatty acids the precipitate was dissolved in water, acidified with acetic acid, and extracted with ether. The fatty acids and the water-soluble components were isolated in the same manner as in the first experiment. The amounts of cleavage products obtained are given in Table II.

The barium salt of the organic phosphoric acid was combined

with a similar barium salt obtained after the polysaccharide had been treated with alcoholic potassium hydroxide and was examined as will be described later.

The glycerol was identified by means of its tribenzoate.

The fatty acids were combined with those obtained in the first experiment and examined as will be described elsewhere.

Examination of Polysaccharide—In an earlier investigation (1) it had been observed that an organic phosphoric acid was split off when the polysaccharide was treated with alcoholic potassium hydroxide; hence the same reaction was carried out in the present case. The crude polysaccharide was suspended in 300 cc. of 1 per cent alcoholic potassium hydroxide and the mixture was refluxed on a water bath for 2 hours. The polysaccharide which remained as an insoluble powder in the alcohol was filtered off and washed with alcohol.

The alcoholic solution was saturated with carbon dioxide and the potassium carbonate which separated was filtered off and discarded. The filtrate was evaporated *in vacuo* to dryness and the residue was extracted with absolute alcohol. A syrup weighing 0.3 gm. was obtained on evaporation of the alcoholic extract. The syrup when heated with acid potassium sulfate gave a strong odor of acrolein, thus indicating the presence of glycerol.

The polysaccharide was dissolved in a little water and the solution, after being neutralized with acetic acid, was mixed with an excess of neutral lead acetate and the precipitate of water-insoluble lead salts of inorganic and organic phosphoric acids was filtered off and washed with water.

The polysaccharide was isolated from the filtrate by the usual basic lead acetate-ammonia procedure, and after dehydration by grinding under absolute alcohol was obtained as a white powder which weighed 4.5 gm. It contained phosphorus and nitrogen.

Analysis—Found, P 4.55, N 1.27

The alcoholic mother liquor and washings which remained after the polysaccharide had been dehydrated were examined as will be described later.

Dephosphorylation of Polysaccharide—In previous experiments we have always found that the phosphorus is removed completely when the polysaccharide, dissolved in 14 per cent ammonium

hydroxide, is heated for about 8 hours in a sealed tube at a temperature of 160–170°. In the present experiment, however, the phosphorus was found to be more firmly combined and it was necessary to heat the polysaccharide, dissolved in 14 per cent ammonium hydroxide, four times in sealed tubes before all of the phosphorus was split off. The first two periods of heating were for 8 hours each at 165–170° and the last two periods were for 6 hours each at 180°.

Isolation of Inosite

After the phosphorus had been split off, the reaction mixture was freed of ammonia and phosphoric acid exactly as described in a preceding paragraph and the solution after being decolorized with norit was concentrated *in vacuo* to a thick syrup. Crystallization began very soon and after 24 hours the crystals were stirred up with cold 60 per cent alcohol, filtered, washed with alcohol, and dried. The yield was 0.7985 gm. and after recrystallization from water by addition of alcohol 0.73 gm. of snow-white prismatic crystals was obtained. The substance gave the Scherer reaction and melted at 225°; there was no depression of the melting point when the substance was mixed with inactive inosite.

Acetylation of the Non-Crystalline Portion of Carbohydrate—The filtrate and washings from the inosite crystals on evaporation to dryness left 1.87 gm. of a non-crystalline residue. This substance was acetylated in pyridine and acetic anhydride and the acetyl derivative was isolated as described before. The derivative which weighed 3.4 gm. was crystallized from 10 cc. of methyl alcohol, and the product was washed with cold methyl alcohol. The filtrate was examined as will be described later. The snow-white crystals weighed 0.746 gm. and on recrystallization from methyl alcohol 0.6 gm. of colorless prismatic crystals was recovered. Heated in a capillary tube, the substance began to sinter at about 80° and on continued heating it fused to a nearly transparent mass which crystallized at 150–160° and then melted gradually at about 190°. The crystalline form appeared to be identical with that of inosite hexaacetate but the peculiar melting point showed that the crystals were not pure inosite hexaacetate and it was thought that the substance might have been incompletely acetylated. In

obtained after the polysaccharide had been refluxed with alcoholic potassium hydroxide, as mentioned in a preceding paragraph, were suspended in water and decomposed with hydrogen sulfide. The filtrate from the lead sulfide was concentrated *in vacuo* to a thin syrup and mixed with 95 per cent alcohol. A slight amount of insoluble matter was filtered off and discarded. The alcoholic solution was strongly acid in reaction and on neutralization with barium hydroxide gave a white amorphous barium salt, 2.64 gm., which was treated with 25 cc. of water. The insoluble portion, 0.4 gm., was evidently barium phosphate, since it gave a strong reaction for inorganic phosphate.

The filtrate was diluted with an equal volume of alcohol, which caused a white amorphous precipitate. The substance which weighed 2.12 gm. was combined with the 0.27 gm. of a similar water-soluble barium salt mentioned earlier and dissolved in 15 cc. of water. The solution was filtered from a trace of insoluble material and diluted with 30 cc. of alcohol. The resulting precipitate was collected, washed with 75 per cent alcohol, and with alcohol. The snow-white amorphous powder, 2.25 gm., was easily soluble in water and on reprecipitation with alcohol was recovered quantitatively.

For analysis the substance was dried at 78° *in vacuo* over dehydrite.

Found, Ba 39.85, P 9.33

The substance gave the Scherer reaction, thus indicating the presence of an inosite phosphoric acid. An attempt was made therefore to hydrolyze the acid and to identify such cleavage products as might be formed.

The barium salt, 2.0 gm., was dissolved in a little water and 20 cc. of 5 per cent sulfuric acid were added. After the barium sulfate had been removed, the solution was refluxed for 3 hours, after which the solution was neutralized with barium hydroxide. The precipitate was centrifuged off and tested for inorganic phosphate but only a trace was present; the organic phosphoric acid had therefore been hydrolyzed to a very small extent.

The barium salt of the organic phosphoric acid was recovered from the centrifugate by adding 2 volumes of alcohol. The white precipitate was collected and washed with 75 per cent alcohol and

with absolute alcohol. After being dried *in vacuo* the snow-white powder weighed 1.5 gm. The substance was reprecipitated from water by addition of alcohol as mentioned above and the recovery was quantitative. The salt was analyzed after it had been dried at 78° *in vacuo.*

Found, Ba 37.10, P 9.15

The substance gave the Scherer reaction and evidently represents a slightly impure sample of barium inosite monophosphate.

The alcoholic mother liquor from the barium salt was examined but the only substance that could be identified was a small quantity of crystalline inosite.

Alcoholic Mother Liquor from the Polysaccharide

The alcoholic mother liquor after dehydration of the crude polysaccharide was acid in reaction. It was concentrated to a thick syrup and the latter was dehydrated a second time by grinding under absolute alcohol. A small amount of alcohol-insoluble substance was filtered off and the filtrate was concentrated *in vacuo* to a syrup. The latter was dissolved in water and neutral lead acetate was added until no further precipitate was obtained. The lead salt was filtered off and washed with water, after which it was decomposed in aqueous suspension with hydrogen sulfide. The filtrate was concentrated *in vacuo* to about 25 cc. and neutralized with barium hydroxide. The water-insoluble barium phosphate that separated was filtered off and the filtrate was diluted with 2 volumes of alcohol, which caused a white amorphous precipitate. The precipitate was washed with dilute alcohol and with alcohol and dried *in vacuo.* It weighed 0.7 gm., and was easily and completely soluble in water. The Scherer reaction was negative. The substance was analyzed after it had been dried at 78° *in vacuo.*

Found, Ba 44.87, P 9.60

These values correspond closely with the calculated composition of barium glycerophosphate.

$C_3H_7O_6PBa$ (307.4). Calculated, Ba 44.70, P 10.08

The results of the examination of the water-soluble barium salts associated with the polysaccharide indicate that two types of or-

ganic phosphoric acids were present, namely inosite monophosphoric acid, and glycerophosphoric acid.

DISCUSSION

The results of the present investigations on the composition of the polysaccharide of the phosphatide fractions isolated from tubercle bacilli residues are difficult of interpretation. However, one fact stands out clearly and that is that the carbohydrates of the two phosphatides did not give identical cleavage products. Furthermore the carbohydrates were quite different from the substance which was formerly described under the name of manninositose. The reason for the variations in composition that we have observed may depend upon one of two things, (*a*) different carbohydrates may be formed by the bacillus or (*b*) the configuration of the carbohydrate may have been changed during the heating and autoclaving of the bacilli in the preparation of tuberculin. We can offer no other explanation, because both carbohydrate fractions had been subjected to the same reactions that in earlier experiments had always led to the isolation of manninositose. The results can only be regarded as curious facts that must be taken into consideration in future investigations on the composition of the polysaccharide components of the phosphatide of the human tubercle bacillus.

SUMMARY

1. The polysaccharide fractions of the phosphatides prepared from bacterial residues from tuberculin manufacture are not identical with the polysaccharide contained in the phosphatide prepared from living tubercle bacilli, Strain H-37.

2. Two polysaccharide fractions isolated from two phosphatide preparations gave different cleavage products.

3. The first polysaccharide gave on dephosphorylation free inosite and a glycoside which on hydrolysis gave mannose and some other reducing sugar.

4. The second polysaccharide gave on dephosphorylation some free inosite and a glycoside which on hydrolysis gave about equal parts of inosite and mannose, together with some other reducing sugar.

5. The reducing sugars other than mannose gave typical glucosazones.

6. Neither of the polysaccharides from the bacterial residues gave the glycoside manninositose.

7. The organic phosphoric acids separated from the polysaccharides were of two kinds: (*a*) an acid corresponding approximately in composition to inosite monophosphoric acid, and (*b*) an acid corresponding in composition to glycerophosphoric acid.

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STUDIES ON β-GLUCURONIDASE

III. THE INCREASE IN β-GLUCURONIDASE ACTIVITY OF MAMMALIAN TISSUES INDUCED BY FEEDING GLUCURONIDOGENIC SUBSTANCES

By W. H. FISHMAN

(*From the Department of Biochemistry, University of Toronto, Toronto, Canada*)

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In an earlier paper (1) from this laboratory methods of preparation and partial purification of extracts of animal tissues containing β-glucuronidase and a method for the quantitative determination of the activity of such extracts were described. More recently, a kinetic study of several factors which affect the hydrolytic action of the enzyme on menthol, borneol, and estriol glucuronides was made (2). These studies were undertaken to provide an experimental approach to an investigation of the metabolism of estriol glucuronide in the body, since it seemed possible that this metabolism might be controlled by a glucuronidase. Before proceeding further it was considered desirable to investigate the rôle of the enzyme in the detoxication mechanisms of the body.

It is well known (3) that the amounts of certain enzymes in microorganisms may be markedly increased by adding substrates for the enzymes to the media in which the microorganisms are grown. However, at the time when the present study was commenced the only observation known to the author which suggested that in mammalian tissue an increase in enzymic activity might be effected by the presence of its substrate was that of Weinland (4) who found sucrase in the blood of a dog following the injection of sucrose.[1]

[1] Since the commencement of this work, Leloir and Muñoz (5) have published figures showing that the power of kidney tissue to oxidize ethanol is considerably increased by previous administration of ethanol to the ani-

Until it has been experimentally demonstrated that β-glucuronidase can catalyze the synthesis *in vitro* of conjugated glucuronides, the probable rôle of the enzyme in effecting the conjugation of toxic substances with glucuronic acid in animal tissues must remain in some doubt. However, it was considered justifiable to assume as a working hypothesis that β-glucuronidase does play such a rôle and therefore it was decided to carry out experiments to show whether or not the oral administration of large amounts of glucuronidogenic substances would induce an increase in the enzyme content of animal tissues.

Experiments upon dogs fed with borneol were first carried out.[2] The results strongly suggested that the administration of borneol had caused a substantial increase in the β-glucuronidase content of several tissues. It was soon apparent, however, that, in the interests of economy and general convenience, it would be necessary to work with smaller laboratory animals in order to obtain a sufficient number of observations to permit definite conclusions to be drawn. Accordingly a micromethod for the estimation of β-glucuronidase activity was devised; this made it possible to carry out experiments on mouse tissue. It was considered that the mouse would be a suitable experimental animal, since it has been shown by Pryde and Williams (11) that it does excrete compounds conjugated with glucuronic acid.

In the present communication the results obtained on dogs fed borneol and on mice fed menthol are reported.

EXPERIMENTAL

Experiments with Dogs—On the termination of the period of experimental feeding the animals were killed either by a humane

mal. It seems possible that this could be ascribed to an increase in the kidney ethanol dehydrogenase in response to increased substrate concentration in the tissues. Yudkin's (6) work on the other hand suggests that the tissues of alcohol-tolerant rats do not have any increased capacity to metabolize alcohol. More recently Lightbody and Kleinman (7) have shown that the liver arginase of rats increases as a result of feeding the animals on a high protein diet, and Klein (8) has demonstrated an increase in liver *d*-amino acid oxidase in rats after thyroid feeding. It seems probable that in both cases the results may be ascribed to an increase in enzymic activity in response to an increased amount of substrate.

[2] Preliminary accounts of experiments on dogs fed borneol have been previously made (9, 10).

killing pistol or by intravenous chloroform injection and the fresh tissues were weighed, minced, and the enzyme extracted with water by 1 hour's vigorous stirring. The number of ml. of water employed in the extraction was always twice the number of gm. of the tissue sample (usually 25 gm.). The mixture was then centrifuged for 15 minutes and the supernatant solution was poured into a graduated cylinder and the volume noted (A). An equal volume of acetone was added and the flocculent precipitate which formed was quickly centrifuged. This precipitate was then extracted with a volume of water equal to (A) and the insoluble matter removed by centrifuging. All extracts of organs other than ovary received another such treatment with acetone. The resulting solution of the enzyme was free of suspended tissue particles. 2 ml. of this final aqueous extract were incubated with 1 ml. of M/15 menthol glucuronide buffered with 0.1 N acetate buffer at pH 5.2 for 8 hours. Control experiments were performed with previously boiled extract. The liberated glucuronic acid was determined in the manner previously described (1). The results are expressed as mg. of glucuronic acid liberated per gm. of tissue.

The enzymic activity of various tissues of four normal dogs was first assayed, thus providing a control group. Each of three experimental dogs was fed 5 gm. of borneol daily in gelatin capsules in the food for 4 or 5 days, 24 hour collections of the urine were made, and the zinc salt of borneol glucuronic acid was then isolated from the urine by Quick's method (12). The daily excretion of the glucuronide usually became constant by the 4th or 5th day; 36 hours after the last feeding the animals were killed. The enzyme was extracted from the various organs and the final extracts were stored in the refrigerator and their activity was assayed as quickly as possible. The results are illustrated in Fig. 1.

In the dogs which had been fed borneol significant increases in β-glucuronidase activity were observed in the liver, kidney, and spleen. In the uterus, pancreas, and ovary no increase could be detected. The Fisher t method for testing the significance of the difference of means has been applied to these results and the value of P is in the case of liver 0.0375, kidney < 0.01, spleen < 0.01, pancreas 0.08, ovary 0.4, uterus 0.23.

Experiments with Mice—The activity of the enzyme in extracts from mouse tissue was determined in the following manner. The

organs were quickly dissected out of the carcass and weighed. Each organ was minced with a pair of fine scissors into a graduated 15 ml. centrifuge tube and a small amount of pumice or fine sand was added. The contents of the vessel were stirred vigorously by means of an air-turbine stirring apparatus for 30 minutes, distilled water being added to make up to the final desired volume when maceration of the tissue appeared to be complete. This volume was regulated within the range of 1.5 to 6.0 ml., depending on the quantity of tissue being extracted. The mixture was centri-

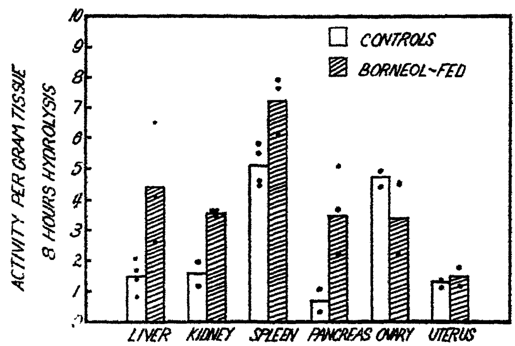

FIG. 1. The response, in terms of β-glucuronidase activity in tissues, to the feeding of dogs with borneol. The activity per gm. of tissue is expressed as mg. of glucuronic acid liberated in 8 hours hydrolysis at 37.5° from menthol glucuronic acid. The means of the activities found in the given tissues for different dogs are indicated by block diagram. Actual activities found are shown as points.

fuged and the volumes of the supernatant liquid and of the precipitate were noted. The activity of the solution was assayed.

Digestion mixtures were prepared in the following manner. In the case of liver, kidney, and spleen, 1 ml. of the extract was diluted in a test-tube with distilled water. The volume selected was such that the amounts of enzyme contained in a unit volume of the diluted extracts were roughly equivalent. This dilution was not necessary before the activity of extracts of the testis, ovary, uterus, and vagina was assayed. 0.40 ml. of the diluted or undiluted extract, depending upon the organ extracted, was transferred with a graduated 1 ml. pipette into each of two test-tubes. One was immersed in vigorously boiling water for 30 to

45 seconds, the other remained at room temperature, and to both were added 0.40 ml. of 0.1 N acetate buffer (pH 5.0) and 0.2 ml. of 0.15 N sodium menthol glucuronidate. The mixtures were incubated for 70 hours at 37.5°, and the glucuronic acid liberated was estimated by the method previously described (2).

A solution of 2 gm. of menthol was emulsified in 15 ml. of water with the aid of 0.5 gm. (approximate) of soap. 3 drops of this solution containing about 20 mg. of menthol were administered orally to mice under light ether anesthesia (13). Each of a group

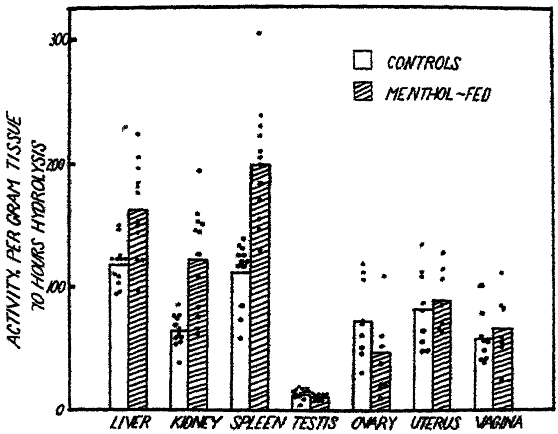

FIG. 2. The response, in terms of β-glucuronidase activity in tissues, to the feeding of mice with menthol. The activity per gm. of tissue is expressed as mg. of glucuronic acid liberated in 70 hours hydrolysis at 37.5° from sodium menthol glucuronidate. The means of the activities found in the given tissues for different mice are indicated by block diagrams. Actual activities found are shown as points.

of twelve male mice and seven female mice received at 9 a.m., 2 p.m., and 5 p.m. 20 mg. of menthol every day for 4 days, and two doses of 20 mg. of menthol 3 hours apart on the 5th day. The animals were killed 3 hours after the last dose and the activity per gm. of tissue was determined and was compared with that of the corresponding tissue of the animals of a control group. The results are illustrated in Fig. 2. It was not possible to show any effect of ether anesthesia on the β-glucuronidase activity of mouse tissue.

Liver, kidney, and spleen of mice previously fed menthol possessed β-glucuronidase activity significantly greater than that of the corresponding tissues of the control animals. No increase in enzymic activity was noted in the testis, ovary, uterus, and vagina. The Fisher t method for testing the significance of the difference of means has been applied to these results and the value of P is in the case of liver < 0.01, kidney < 0.01, spleen < 0.01, testis 0.49, ovary 0.1, uterus 0.8, vagina 0.45.

DISCUSSION

It is clear that the β-glucuronidase content of several tissues in the dog and mouse can be increased by the administration of glucuronidogenic substances to the animals. If it is justifiable to assume that the conjugation of toxic substances with glucuronic acid in animal tissues is catalyzed by the enzyme, then the results provide clear cut evidence of an increase in the enzyme content of animal tissues in response to an overloading of the body with an excess of the substrate of the enzyme; and in view of the results of Leloir and Muñoz, Klein, and of Lightbody and Kleinman, to which reference has already been made, one is inclined to believe that adaptation of the enzyme systems in animal tissues to cope with unusual demands upon them may be a general phenomenon. Although it is fully appreciated that until the synthetic activity of the enzyme has been demonstrated the justification of the initial assumption must remain in some doubt, it is difficult to see how the experimental results could be explained unless this assumption is a correct one. In the remainder of this discussion it will be assumed that β-glucuronidase does in fact catalyze the synthesis of conjugated glucuronides.

Hemingway, Pryde, and Williams (14), on the basis of experiments in which surviving tissues of the dog were perfused with solutions containing glucuronidogenic substances, concluded that the liver is "the main, if not the only," site of detoxication by conjugation with glucuronic acid. However, the demonstration by Oshima (15) that other organs besides the liver contain high concentrations of β-glucuronidase, and the confirmation of this fact in the present work, suggest that the process of glucuronic acid conjugation is by no means entirely confined to that organ. The increase in β-glucuronidase activity which has been shown to

occur in other organs besides the liver following the administration of glucuronidogenic substances adds considerable support to this suggestion. Nevertheless, by reason of its greater total bulk, it is probable that the liver is, quantitatively, the most important site of detoxication. It might be pointed out in this connection that these results indicate that the determination of the amount of glucuronic acid in the urine following administraton of glucuronidogenic substances which has been suggested by Salt (16) as a test of liver function might give somewhat unreliable results. The results obtained in the case of the sex organs deserve special comment. In both the dog and mouse, administration of the glucuronidogenic substance resulted in no increase in the β-glucuronidase activity in the ovary and uterus, while in the mouse no increase of activity was observed in the testes and vagina.[3] A possible explanation of these results might be that the enzyme in these sex organs is not concerned primarily in the general detoxication processes of the body, but has some function connected with the metabolism or transport of the sex hormones. Experiments designed to investigate this possibility are at present in progress.

SUMMARY

1. An increase in the β-glucuronidase activity of various tissues of the dog and mouse was induced by the previous oral administration of glucuronidogenic substances.

2. On the assumption that β-glucuronidase catalyzes the synthesis of conjugated glucuronides in mammalian tissue these results indicate that an increase has occurred in the enzyme content of animal tissues in response to an overloading of the body with an excess of the substrate for the enzyme.

The author is greatly indebted to Professor G. F. Marrian for many helpful suggestions in this investigation and also in the preparation of the manuscript for publication.

Grateful acknowledgement is made to Miss Dorothy Skill for technical assistance.

[3] An experiment on a single male dog fed borneol indicated that a considerable increase in the β-glucuronidase of the testes had occurred. It is doubtful, however, if any significance can be attached to a single result.

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THE RELATIVE ANTIKETOGENIC ACTIVITY OF GLUCOSE, GLYCINE, AND ALANINE

By ARNE N. WICK, EATON M. MacKAY, HERBERT O. CARNE, and HARRY M. MAYFIELD

(*From the Scripps Metabolic Clinic, La Jolla, California*)

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Glycine and *dl*-alanine have been shown to be as efficacious as glucose in the formation of hepatic glycogen, although there is considerable difference in the rate at which glycogen is deposited after the feeding of these substances (1). Being equally good glycogen formers, they should have the same antiketogenic activity, but Butts *et al.* (2) have reported that *dl*-alanine is much better than glycine as an antiketogenic agent. The relation of the difference in the rate of glycogen formation after the administration of these substances to the method used by Butts *et al.* to measure antiketogenic activity suggests the reason for the apparent discrepancy between glycogenic and antiketogenic activity. These investigators measured antiketogenic activity by determining the influence of the administered substance upon the excretion of acetone bodies in the urine of fasting rats which were fed sodium acetoacetate. Because of renal threshold changes, urine excretion rates alone may lead to erroneous conclusions (3). But even assuming that the urine rate correctly reflected the level in the blood, the degree of ketosis as measured by the blood level in these rats was the sum of the fasting ketosis plus the acetoacetic acid fed plus the ketogenic action of any of the fed alkali freed to exert an alkalosis action by the catabolism of the acetoacetic acid with which it was combined. Under such circumstances the production of ketone bodies would be irregular and the blood concentration variable. Substances giving the same amount of glycogen could then affect the renal excretion of ketone bodies very differently if the time of glycogen formation differed. The more slowly a substance produced glycogen the less its antiketogenic effect might appear

to be, for the highest blood acetone body concentrations and in all probability urine acetone body excretion would be reached soon after the sodium salt was fed when the formation of glycogen was at a minimum. This could easily explain the diverse effect of glycine which produces glycogen very slowly. In an attempt to circumvent this difficulty, we have examined the antiketogenic action of the compounds in question by determining their influence upon not only the acetone bodies in the urine but their concentration in the blood of fasting rats. We assume that acetone body production and utilization are approximately constant in these rats and that any decrease in the blood level is due to a reduction in their rate of production but we have no direct proof of this. In the present experiments, this point is immaterial, for the effect is the same with the various substances, regardless of how it is brought about.

After a period on a low protein diet, albino rats were fasted. Such a diet leads to a higher fasting concentration for the blood acetone bodies (4). Urine collections were commenced on the 2nd day of fasting. Urine collections were made over 24 hour periods and the substances being studied were generally administered at the beginning and middle of the urine collection periods. Exceptions to this are listed in the foot-notes to Table I. Blood acetone body determinations were always carried out on blood collected at the end of the urine collection periods. Since taking these samples involved the sacrifice of an animal, making blood acetone body determinations reduced the number of rats in a group on which urine was being collected, as noted in Table I.

The rats were fed by stomach tube, the solutions[1] being measured in 5 cc. syringes and the doses given to the nearest 0.1 sq.dm. of body surface. Body surface was calculated from the gross body weight by the formula of Carman and Mitchell (5). As usual, doses administered and the excretion rates are given in terms of

[1] The glucose used was the C.P. anhydrous grade of the Pfanstiehl Chemical Company. Glycine was purchased from E. R. Squibb and Sons. It contained 0.8 per cent ash and 0.25 per cent moisture. Pfanstiehl's *dl*-alanine was used, and, according to our analysis, it contained 15.71 per cent nitrogen, 0.22 per cent ash, and 0.58 per cent moisture. The *l*(+)-alanine was obtained from F. Hoffmann-La Roche and Company, A. G., Basle, Switzerland, in 1935. This preparation has a moisture content of 0.02 per cent and contained no ash. The $[\alpha]_D^{28} = +2.5°$ in aqueous solution.

"body surface," because the total resting metabolism, activity of the kidney, intestinal absorption rates, organ weights and composition, and other physiological measurements bear a less variable relationship to such a mathematical function of the body weight than other measurements of body size (6). Urine was collected under light mineral oil. No attempt was made to empty the bladders of the experimental animals completely except at the beginning and end of a series of collections. Urine acetone bodies were determined by Van Slyke's method (7). Blood acetone bodies were determined by the micro adaptation of Van Slyke's method, introduced by Barnes and Wick (8).

Our results are summarized in Table I. Antiketogenic activity is measured by the reduction in the ketonuria and the decrease in the level of the blood acetone bodies. The latter are a better measure of ketogenesis, for the ketonuria simply reflects the level of acetone bodies in the blood and, since they are treated as threshold substances by the kidney, the urine rates do not always faithfully reflect the blood level. However, in most of our experiments the ketonuria figures lead to the same conclusions as a study of the changes in the blood levels. There is variability, as might be expected in such experiments, but equivalent (based on the number of carbon atoms) amounts of glucose, glycine, and *dl*-alanine always have practically the same antiketogenic activity. This may be seen in all of the individual experiments (Table I) as well as from the weighted averages of all the data. The average blood acetone body concentrations are 56 mg. per cent for the controls, 20 mg. per cent for the glucose-fed, 22 mg. per cent for the glycine-fed, and 19 mg. per cent for the *dl*-alanine groups. This is the result which would be expected from the similar glycogen-forming ability of these three compounds (1). Since glycine is a slower glycogen former than the other two (1), it is not surprising that it may at times be slow in exerting its antiketogenic effect (Experiment 1, third group). The manner in which a given quantity of material is administered may determine to some extent the antiketogenic action it exerts. In Experiment 2, the same amount of glucose has the same antiketogenic effect whether it is given in a single dose or at the beginning and middle of the periods. Glycine and *dl*-alanine (Experiment 2, fourth to seventh groups) on the other hand have a definitely greater effect when administered

TABLE I

Relative Influence of Glucose, Glycine, and Alanine upon Fasting Ketosis of the Rat As Measured by Ketonuria and Level of Acetone Bodies in Blood

Experiment No.*	Average body weight	Average body surface	Period on special diet† before fasting	Solution fed in doses‡ of 1 cc. per sq.dm. body surface	Urine acetone bodies per sq.dm. body surface per day					Blood acetone bodies§ at end of				
					1st day	2nd day	3rd day	4th day	5th day	1st day	2nd day	3rd day	4th day	5th day
	gm.	sq.dm.	days		mg.	mg.	mg.	mg.	mg.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
1 (7) ♀, Diet 1	206	4.0	22	Water	25.3	26.7	16.5	8.5	2.0	58	51	36	39	55
	205	4.0	22	0.3 M glucose	7.2	2.7	0.0	0.3	0.0	28	21	20	9	11
	206	4.0	22	0.9 " glycine	15.5	7.3	4.0	0.4	0.0	60	32	16	17	18
	207	4.0	22	0.6 " *dl*-alanine	7.0	1.3	0.0	0.0	0.0	30	25	9	12	10
	208	4.0	22	0.6 " *l*(+)-alanine	3.6	1.7	0.0	0.0	0.0	10	22	12	17	14
2 (6) ♀, Diet 1	208	4.0	25	Water	10.8	24.0	11.4	1.2	5.7		66	58	40	38
	207	4.0	25	0.3 M glucose	4.1	0.0	0.0	0.0	0.0		20	8	20	19
	205	4.0	25	0.6 " "	1.2	0.0	0.0	0.0	0.0		14	12	19	20
	208	4.0	25	0.9 " glycine	13.2	1.6	1.0	0.0	0.0		22	21	16	13
	208	4.0	25	1.8 " "	6.5	2.6	1.8	0.0	2.3		24	46	16	43
	210	4.0	25	0.6 " *dl*-alanine	0.4	0.0	0.0	0.0	0.0		12	12	20	12
	208	4.0	25	1.2 " "	5.0	0.8	8.2	0.0	0.0		34	36	30	15
3 (8) ♀, Diet 1	177	3.6	26	Water	35.7	34.2	17.7	5.9		67	64	60	65	
	180	3.6	26	1.0 M glucose	12.8	0.4	0.5	0.0		13	8	9	8	
	180	3.6	26	2.0 " *dl*-alanine	9.8	0.5	0.5	0.0		27	9	8	6	
4 (6) ♂, Diet 2	270	4.7	16	Water	7.6	38.8	27.3	1.5			92	87	44	
	272	4.8	16	0.25 M glucose	6.6	9.3	2.5	0.4			22	25	15	
	274	4.8	16	0.75 " glycine	10.2	10.0	0.6	0.9			24	19	21	

5 (10) ♂, Diet 2	264	4.7	8	Water	25.8	20.0	9.4	10.5	24.8	58	94	49	53	40
	261	4.6	8	0.1 M glucose	9.9	4.4	0.0	0.0	0.0	37	54	17	18	34
	247	4.5	8	0.2 " *dl*-alanine	8.8	7.1	0.0	0.0	0.0	37	46	15	19	23
6 (7) ♂, Diet 1	199	3.9	15	Water						55	55	53	56	35
	198	3.9	15	0.3 M glucose						46	29	10	17	14
	190	3.8	15	0.6 " *dl*-alanine						18	23	14	7	17
7 (6) ♀, Diet 2	201	3.9	15	Water						75	72	61		
	199	3.9	15	1.0 M glucose						26	12	9		
	199	3.9	15	3.0 " glycine						21	16	8		
	197	3.8	15	2.0 " *dl*-alanine						21		11		

* The figures in parentheses denote the number of rats in each group of the experiment.

† Diet 1 was composed of sucrose 45, casein 5, standard Osborne and Mendel salt mixture 5 (9), brewers' yeast 5, cod liver oil 5, and Crisco 35. The composition of Diet 2 was the same except that Harris yeast extract, 2 parts, was used in place of brewers' yeast and the sucrose content was 48.

‡ This dose of solution was administered at the beginning and middle (2 cc. per sq.dm. of body surface per day) of the 24 hour periods of urine collection except in Experiments 2 and 5. In the third, fifth, and seventh groups of Experiment 2 single doses were used at the start of the collection period, and in Experiment 5 three doses were given, being administered at the 0, 5th, and 11th hours of each urine collection period.

§ The figures for blood acetone bodies represent averages of determinations made on two rats except in the case of those for the 1st, 2nd, and 4th days of Experiments 1 and 2 and the 1st, 3rd, and 4th days of Experiment 6 for which the determinations were carried out on the blood of a single rat.

in divided doses 12 hours apart than in a single dose every 24 hours. It is possible that there is a significant loss in the urine with a single large dose.

It is of interest (Experiment 1, fourth and fifth groups) that *l*(+)-alanine has practically the same antiketogenic activity as *dl*-alanine. It has been reported (2) to be a better glycogen former than *dl*-alanine but this may be a question of the rate of formation rather than a difference in amount (1).

SUMMARY

Glucose and equivalent amounts of glycine, *dl*-alanine, and *l*(+)-alanine exert the same antiketogenic action in fasting rats when measured by the effect on the level of acetone bodies in the blood and the amount of these substances excreted in the urine.

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STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

IX. THE THROMBOPLASTIC PROTEIN FROM LUNGS*

By SEYMOUR S. COHEN† AND ERWIN CHARGAFF

(*From the Departments of Biochemistry and Surgery, College of Physicians and Surgeons, Columbia University, New York*)

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The classical theory of blood coagulation (1) postulates two phases:

$$\text{Prothrombin} \xrightarrow{\text{Ca}^{++},\ \text{thromboplastin}} \text{thrombin}$$

$$\text{Fibrinogen} \xrightarrow{\text{thrombin}} \text{fibrin}$$

The factor which together with calcium ion is responsible for the activation of prothrombin to thrombin has variously been called thromboplastin, thrombokinase, thrombozyme, cytozyme, etc. It appears to occur in most animal tissues, from which it is released whenever a wound is produced, thereby giving rise to extravascular clotting. Its presence in the extremely fragile blood platelets probably is the principal cause of intravascular coagulation.

A consideration of the chemistry of the thromboplastic effect has to start from a number of facts which have partially been known for a long time. (1) Alcoholic or ethereal cell extracts contain a thermostable factor (called "zymoplastic substance" by its discoverer Schmidt (2)) which functions as a clotting activator. The substance responsible for this effect is a phosphatide (3–6) and appears to belong to the cephalin group (5, 7).

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† This report is from a dissertation submitted by Seymour S. Cohen in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

Blood platelets contain an extremely active cephalin fraction (8). (2) Aqueous tissue extracts contain a thermolabile factor of apparently protein nature which, although much more potent than cephalin,[1] activates the clotting of blood in a manner essentially similar to that of this phosphatide. Wooldridge (9), one of the earliest workers in the field, already considered this substance as a phosphatide-protein complex. There are three possible explanations for the much greater potency of the thromboplastic protein as compared with isolated cephalin: (*a*) the presence in it of a particularly active cephalin, (*b*) an effective orientation of the cephalin molecules on the surface of a specific protein, (*c*) the activity of the phosphatide-free protein component.

Among the more recent investigations of the thromboplastic protein a study by Mills (10) may be mentioned as well as a short note by Fischer and Herrmann (11) which appeared while our work was in progress. The present paper comprises a study of the preparation and properties of the thromboplastic protein from lungs, together with some data on its immunological behavior. Additional information on the chemical composition of this substance will, it is hoped, be reported later.

EXPERIMENTAL

Isolation of Thromboplastic Protein from Lungs

In a typical experiment, 1860 gm. of lungs from freshly killed cattle were minced and extracted in the refrigerator with 3 liters of physiological saline for 12 hours. The material was filtered through a canvas bag, when 1950 cc. of a turbid solution were obtained. The addition of enough saturated $(NH_4)_2SO_4$ solution to correspond to 10 per cent saturation resulted in the formation of a precipitate which after chilling was centrifuged off and discarded. The supernatant was brought to 30 per cent saturation with $(NH_4)_2SO_4$ and the mixture cooled overnight.[2] The resulting precipitate was separated from the inactive supernatant by

[1] For a comparison of the activities of platelet phosphatides and breast muscle extract, see Figs. 1 and 3 in (8).

[2] The material precipitable between 10 and 30 per cent saturation with $(NH_4)_2SO_4$ was found by activity tests with chicken plasma as substrate (8) to contain by far the largest proportion of the thromboplastic protein of lung tissue.

centrifugation in a refrigerated angle centrifuge at 4000 R.P.M., taken up in 200 cc. of physiological saline, and dialyzed against running water for 12 hours. The complete removal of salts by dialysis was sufficient to produce precipitation of the protein.[3] The precipitate was, after separation from the inactive supernatant, frozen in a solid CO_2-alcohol mixture and dried in the frozen state in a high vacuum. The completely dehydrated substance, after exhaustive washing with ice-cold acetone and drying *in vacuo*, weighed 8.5 gm. and was a light tan-colored powder. In the customary clotting test (8) the addition of 0.06

TABLE I

Thromboplastic Protein Preparations from Beef Lungs

Preparation No.	P	N	S	Activation of plasma clotting	
				Thromboplastin in 0.1 cc. plasma	Clotting time*
	per cent	*per cent*	*per cent*	*mg.*	*min.*
1	1.04	11.8		0.03	3(80)
2	1.02	11.7			
3	0.83	12.0			
4	0.74	12.7	0 94	0.03	6(130)
5	0.95			0.06	6(>120)
6	0.87			0.04	6(>70)
7	0.79	12.3	0.93		
8	0.81	12.2	1.01		

* The normal clotting times of the chicken plasma preparations used are given in parentheses.

mg. of this material in 0.03 cc. of saline to 0.1 cc. of chicken plasma (clotting time over 2 hours) produced a clotting time of 6 minutes at 30°. Analytical data and thromboplastic activities of a number of preparations of the protein are given in Table I.

The preparations obtained in the manner here described could be stored for a long time without loss of activity. It should be pointed out that in the process of drying the thromboplastin preparations became quite insoluble in water and dilute salt solu-

[3] In several experiments the addition of a few drops of dilute acetic acid to the dialyzed mixture was found to make the precipitation of the thromboplastic protein more complete.

tions. The thromboplastic potency of aqueous emulsions of the compounds was, however, preserved. Whereas attempts to effect the removal of water from the wet preparations by means of acetone led to inactive products, the treatment of the dry powders with acetone did not affect their activity. Treatment with alcohol resulted in the loss of thromboplastic potency. In a number of experiments which will be discussed later in this paper the drying process was omitted altogether, and the precipitated proteins were, after the dialysis, redissolved in buffer solutions of the desired pH. It might be noted that the thromboplastic activity of the proteins was not injured by solution at pH 8.8 and reprecipitation at pH 5.1. The addition of 0.03 cc. of a 1 per cent solution of the protein which had undergone this treatment to 0.1 cc. of chicken plasma (normal clotting time more than 2 hours) produced a clot in 6 minutes at 30°.

Isolation of Phosphatides from the Thromboplastic Protein

The most characteristic chemical property of the thromboplastic protein is its phosphorus content. In view of the well known activity of cephalin in blood clotting it appeared of interest to study methods for the isolation of phosphatides from the thromboplastic protein. An attempt to bring this about by precipitation of the lipids as a complex with a basic dye (12) failed. By extraction with ether no phosphatides were removed. When an aqueous emulsion of the thromboplastic protein was shaken with $CHCl_3$ for 25 hours, only 20 per cent of the total P went into the $CHCl_3$. Subsequent extraction of the dried protein residue with boiling $CHCl_3$ for a week yielded an additional 10 per cent of the total P originally present.

An effective method for the removal of most of the lipid P was found to be the continuous extraction of the thromboplastic protein with boiling alcohol-ether. In a typical experiment 8.00 gm. of a thromboplastic protein preparation containing 0.92 per cent P (*i.e.* a total of 73.6 mg. of P) was extracted with 200 cc. of boiling alcohol-ether (1:1) for 7 days. The filtered extract was evaporated under nitrogen; the lipids were redissolved in $CHCl_3$, filtered, evaporated, and dried. They weighed 1.46 gm. and contained 3.27 per cent P (*i.e.* a total of 47.7 mg. of P). The extracted protein weighed 6.50 gm. and contained 0.38 per cent P

(*i.e.* a total of 24.7 mg. of P). The purine N of this substance (13) was found to be 0.17 per cent, which would correspond to a nucleic acid P content of 0.15 per cent. 65 per cent of the P present in the thromboplastic protein was thus accounted for in the extracted phosphatides, and the presence of 13 per cent in the form of nucleic acids was made probable.

Another method for the isolation of phosphatides from the protein is exemplified by the following experiment. The thromboplastic protein after dialysis was emulsified in saline, adjusted to pH 5.1, and centrifuged. The washing was repeated twice until no more colored impurities were removed. The protein was then dissolved in 0.1 M borate buffer of pH 8.8 to give an approximately 1 per cent solution which was dropped with stirring into 4 volumes of boiling alcohol-ether (1:1). After 2 hours at room temperature the coagulated protein was removed, the filtrate was evaporated to dryness *in vacuo*, the lipid residue dissolved in $CHCl_3$, filtered, and again evaporated.

In a phosphorus balance experiment of this type 450 cc. of a solution of the thromboplastic protein at pH 8.8 containing 0.104 mg. of P per cc. (*i.e.* a total of 46.9 mg. of P) were slowly poured into 2 liters of boiling alcohol-ether (1:1). The dried protein residue, after washing with alcohol-ether and with ether, weighed 2.88 gm. and contained 0.40 per cent P (*i.e.* a total of 11.5 mg. of P). The purine N of the protein residue was found to be 0.35 per cent, corresponding to a nucleic acid P content of 0.31 per cent. Only 5 per cent of the P present in the thromboplastic protein was, therefore, unaccounted for.

The chemical composition of the lipids isolated from the thromboplastic protein by the methods here described will form the subject of a future communication.

Isolation of Protein Moiety of Thromboplastic Protein

The mechanism of action of the thromboplastic protein in blood clotting is not well understood. It appeared of obvious interest to attempt the isolation under as mild conditions as possible of the protein constituent of the thromboplastin free of P-containing substances. For this purpose use was made of the reaction between protamines and phosphatides, previously studied in this laboratory (14). A similar procedure has recently been

applied by Warburg and Christian (15) for the removal of nucleic acid from the protein of the oxidizing fermentation enzyme. In order to insure the simultaneous removal of phosphatides (*e.g.* lecithin, etc.) less acidic in nature than cephalin, the reaction was carried out in alkaline solution.

The thromboplastic protein, after precipitation between 10 and 30 per cent saturation with $(NH_4)_2SO_4$, was dissolved in saline and dialyzed against running water for 16 hours. The precipitate which had separated was dissolved in 0.1 N NaOH, an aqueous solution of salmine sulfate was added until no further precipitation took place, and the reaction was adjusted to pH 10. The mixture was stirred at 0° for 2 hours, the precipitate was centrifuged off, and the supernatant filtered. The filtrate was brought to 30 per cent saturation with $(NH_4)_2SO_4$ and adjusted to pH 9. The precipitated protein was, after cooling, removed by centrifugation. One portion was dissolved in physiological saline, in which it was much more easily soluble than the original thromboplastic protein, for activity tests; the other was treated with acetone and dried for analysis.

This protein was found entirely free of P; the procedure removed not only the phosphatides but also the nucleic acid. This protein, free of P-containing substances, had no thromboplastic activity whatever.

Electrophoretic Experiments

Phosphatides—The type of bond between phospholipids and proteins in the compounds occurring in nature commonly designated as lipoproteins is not known. Certain synthetically prepared lipoproteins clearly are true salts between basic proteins and acidic phosphatides (14, 12). It was hoped that the behavior of the thromboplastic protein in an electric field would shed light on this problem. As a first approach experiments on the separation of phosphatide mixtures by electrophoresis were carried out. The electrophoresis apparatus used was essentially the improved arrangement of Theorell (16).[4] It was found advantageous to place the various compartments, which with the exception of the two central electrophoresis glass cells were made of strong ma-

[4] We are indebted to Mr. F. Rosebury of the Department of Biochemistry for the construction of the electrophoresis apparatus.

hogany, on wheels in order to facilitate the assembly of the apparatus. Parchment paper impermeable to phosphatides was placed between the glass cells and the buffer reservoirs. The two center glass cells were separated from each other by Whatman filter paper No. 54, which was found most suitable for the free passage of migrating phosphatides. At the start of the experiments all compartments were filled with buffer solution with the exception of the glass cell nearest to the cathode, which received the solution to be subjected to electrophoresis.

For the experiments here described a mixture, freshly prepared from beef brain by the usual procedures, of lecithin and cephalin free from cerebrosides and sphingomyelin was employed. Analysis showed P 3.2 per cent, N 1.8, amino N 1.5. This material was emulsified in 0.1 M acetate buffer of pH 5.1 to give a 1 per cent emulsion.[5] 400 cc. of this emulsion were dialyzed in viscose casings for 24 hours against the 30 liters of 0.1 M acetate buffer of pH 5.1, which were subsequently used in the buffer reservoirs of the electrophoresis apparatus. For electrophoresis 400 cc. of the dialyzed 1 per cent phosphatide emulsion were introduced into the cathode cell,[6] and a direct current of 220 volts was placed across the silver-silver chloride electrodes. The experiment lasted 2 hours, during which time the current had risen from 1.25 to 1.60 amperes. Soon after the start of the electrophoresis the material could be seen moving in a sharp boundary away from the cathodic parchment membrane into the anode cell, where it coated the anodic membrane. At the end of the experiment the contents of both cells were quantitatively removed and 2 volumes of acetone were added to each of the two emulsions. The precipitates were cooled, centrifuged off, dried, and extracted with ether. The phosphatides were reprecipitated from their filtered ethereal solutions by means of acetone.

[5] This pH was chosen in view of the experiments on the electrophoresis of the thromboplastic protein, the isoelectric point of which appears to be near pH 5.1. At this reaction, therefore, an electrophoretic separation of the stationary thromboplastic protein from migrating phosphatide impurities ought to be possible. Since this pH is intermediate between the theoretical isoelectric points of lecithin and cephalin, they should move in opposite directions in an electric field.

[6] In the following paragraphs the glass cells nearest the cathode and anode will be designated as cathode cell and anode cell respectively.

The analytical data are contained in Table II, where the "Control" represents a sample of the initial phosphatide mixture which had undergone the same reprecipitation process without being subjected to electrophoretic separation. It can be seen that, although considerable concentration of cephalin in the anode cell had taken place, some of the lecithin also had migrated in the same direction. The theoretical isoelectric point of lecithin is at pH 7.5 (17). The presence of free fatty acids has been shown to bring about a considerable depression of the isoelectric point

TABLE II

Electrophoresis of Lecithin-Cephalin Mixture at pH 5.1

	N	Amino N	Amino N	P	N:P
	per cent	*per cent*	*per cent of total N*	*per cent*	
Control	1.61	1.08	67	3.26	1.1:1
Cathode cell	1.68	0.90	53	3.04	1.2:1
Anode cell	1.53	1.24	81	3.12	1.1:1

TABLE III

Electrophoresis of Thromboplastic Protein at pH 8.8

	N	P	N:P
	mg. per cc.	*mg. per. cc.*	
Before electrophoresis	3.27	0.36	20.2:1
Cathode cell	2.06	0.22	20.7:1
Anode cell	1.14	0.13	19.5:1

of lecithin (18). It is conceivable that in the present experiment the admixture of the acidic cephalin produced a similar effect.

Thromboplastic Protein—When a 1 per cent emulsion of the thromboplastic protein in 0.1 M acetate buffer of pH 5.1 was subjected to electrophoresis in the manner described, no movement of material could be observed. For the duration of the experiment (2 hours) the current varied between 1.10 and 1.40 amperes. No evidence for the dissociation of the compound nor for the migration of contaminating substances could be found. A small amount of protein present in the anode cell at the termination of the experiment was doubtless due to filtration, since it had the

same analytical composition as the material originally placed in the cathode cell. The thromboplastic activity and analytical composition of the protein which had remained in the cathode cell were not affected by the electrophoresis.

In another experiment the possible dissociation of the thromboplastic protein into phosphatide and protein at alkaline pH was investigated. An approximately 2.5 per cent solution of the thromboplastic protein in 0.1 M borate buffer of pH 8.8 was subjected to electrophoresis at about 4 amperes for 1½ hours. As can be seen from the data contained in Table III, the decrease in the N:P ratio of the material transferred into the anode cell as compared with that remaining in the cathode cell is so slight that no appreciable liberation of phosphatides due to dissociation of the thromboplastic protein could have taken place.

Immunological Experiments

The experiments reported in the preceding pages showed the phospholipids present in the thromboplastic protein to be very firmly bound. It appeared of interest to study the antigenic properties of this protein which may be considered as a comparatively well defined example of a naturally occurring lipoprotein. The part played by lipids in immunity reactions is still a matter of dispute.

Rabbits were used for the immunization experiments. The production of immune sera against the thromboplastic protein was found to be complicated by the fact that the compound was quite toxic when injected intravenously. This was to be expected on the basis of results obtained by previous investigators. The intravenous injection of 8 mg. of the protein in 4 cc. of physiological saline produced death in one animal by thrombosis in the vena cava. In general, 4 mg. of the protein in 2 cc. of saline were tolerated, although even this amount occasionally produced lethal effects.

The material used for the injections consisted of a very fine 0.2 per cent suspension of the thromboplastic protein in physiological saline containing 0.01 per cent of ethyl mercurithiosalicylate. The protein preparation had been dried in the frozen state, as described above, and washed with chilled acetone. In all experiments the quantitative agglutination tests were carried

out according to the method of Heidelberger and Kabat (19). The antigen suspensions used in the quantitative determinations were prepared by treating the thromboplastic protein with 100 to 200 parts of saline in a mortar, and filtering once or twice through cotton with gentle suction. These suspensions were stable for at least a half hour, and contained between 0.25 and 0.5 mg. of N per cc.

Three series of injections were given.[7] In all cases the individual dose amounted to 4 mg. of protein in 2 cc. of saline. In the first series five injections were administered to each of five animals in the course of 8 days. The thromboplastic action of the protein caused extensive thrombosis of the ear veins, and therefore in the two following series the animals received the material intraperitoneally. In the second series twelve doses were administered in the course of 3 weeks. The third series of injections comprised twenty doses in the course of 5 weeks. After each series blood for testing was removed by heart puncture. During the entire experiment each animal, therefore, had received a total of 148 mg. of the thromboplastic protein. The increase in antibody N during the treatment is shown in the following example: I-AS-1 contained 0.10 mg., II-AS-1 0.21 mg., III-AS-1 0.24 mg. of antibody N per cc.

The analytical results obtained after the third series of injections are contained in Table IV.

The protein residue obtained by extraction of the thromboplastic protein with boiling alcohol-ether (1:1) was completely devoid of precipitating properties when tested with the antisera described above. The same was true of the compound prepared by dropping a solution of the thromboplastic protein at pH 8.8 into alcohol-ether (1:1) at 30°. Repetition of this experiment at −15° yielded a protein which was almost inactive as precipitant. On the other hand, the protein obtained by shaking the thromboplastic protein in veronal buffer of pH 7.0, as described in Paper X (20), had retained about half of its activity. The mildest method yet devised for the preparation of a protein free of phosphatides, viz. the displacement of the phosphatides in the thromboplastic

[7] The antisera obtained after each of the three series of injections are designated I-AS, II-AS, III-AS respectively, followed by the number of the animal.

protein by heparin (20), yielded a compound in which the combining power with the antibody was completely unimpaired. This is of interest, since it shows that the phosphatides are not essential

TABLE IV

Agglutination of Suspensions of Thromboplastic Protein by Rabbit Antisera

The volume of antigen suspensions was 1.0 cc. in each case.

Serum	Volume of serum used	Total N	Antigen N	Antibody N
	cc.	*mg.*	*mg.*	*mg. per cc.*
Normal	1.0	0.49	0.49	
	2.0*	0.48	0.48	
III-AS-1	1.0	0.69	0.49	0.24
	2.0*	0.52	0.48	
III-AS-2	1.0	0.66	0.49	0.20
	2.0*	0.51	0.48	
III-AS-3	1.0	0.65	0.49	0.18
	2.0*	0.50	0.48	
III-AS-4	1.0	0.71	0.49	0.23
	2.0*	0.49	0.48	

* Supernatant.

TABLE V

Thromboplastic Activity of Antigen-Antibody Precipitates

Serum used*	Clotting time† in dilutions of			
	1:1	1:2	1:4	1:8
	min.	*min.*	*min.*	*min.*
Normal (0.23 mg. N per cc.)	6	6	12	15
II-AS-1 (0.38 " " " ")	3	3	6	9
II-AS-2 (0.36 " " " ")	3	3	6	9
Normal (0.49 " " " ")	3	6	9	15
III-AS-1 (0.69 " " " ")	3	3	6	9

* The figures in parentheses indicate the N content of the original suspensions of antigen-antibody precipitates.

† Clotting time of control >120 minutes.

for the capacity of the thromboplastic protein to combine with antibodies.

It was of interest to test the precipitates between antigen and antibody for thromboplastic activity. The precipitates obtained

by the addition of 1 cc. of a suspension of the thromboplastic protein in physiological saline to 1 cc. of antiserum or normal serum were handled as in the quantitative determination of antibodies (19). They were then resuspended in 1 cc. of saline and tested at 30° by adding 0.03 cc. of various dilutions of these suspensions to 0.1 cc. of chicken plasma (8). The experimental results are given in Table V. As a control the clotting activity of a specific precipitate of egg albumin with a rabbit antiserum against egg albumin was tested. This compound, for which we are indebted to Dr. H. P. Treffers of this College, had no activity when tested with chicken plasma.

DISCUSSION

The thromboplastic protein described in the present communication may be considered a lipoprotein. The study of this lipoprotein was made attractive by the fact that it is susceptible to biological assay. Both a protein and a phosphatide component are required for maximal thromboplastic action. The protein component, after removal of the phosphatides, is inactive, and the free phospholipids, an exceedingly complex mixture, show no more than the activity usually exhibited by cephalin. It is not possible to say at present in what manner the phosphatides are linked with the protein. Recent studies from this laboratory (14, 12) have dealt with lipoproteins, which are true salts. It appears doubtful whether the thromboplastic protein falls into this class. The result of the electrophoresis experiments appears to disprove the assumption that the lipids are present as impurities only. The non-dissociability of the thromboplastic protein at pH 8.8 speaks against the supposition of a simple salt-like combination between the protein and the phospholipids. The lack of more detailed structural information is by no means unique with lipoproteins; it extends to most compounds between proteins and substances acting as prosthetic groups.

The fact that mixtures of alcohol and ether in contrast to ether alone remove the phosphatides from the thromboplastic protein, a phenomenon frequently encountered in work with animal and plant tissues and with bacteria, must be due to a destructive action of the alcohol on those groupings in the protein which are responsible for the binding of the phosphatides.

The immunological experiments showed that the thromboplastic protein was able to produce antibodies in rabbits. The specificity, however, seemed to rest entirely with the protein component of this substance: the thromboplastic protein in which the phosphatides had been displaced by heparin (20) was agglutinated by an antiserum against the intact protein. It should be pointed out that the complex between the thromboplastic protein and its antibody was active in the promotion of clotting; it was in fact more active than the free antigen. Whatever molecular configurations contribute to the physiological activity of the protein, it is evident that these groupings are not firmly combined or "covered" by linkage of thromboplastin to antibody.

The authors wish to express their appreciation to Dr. M. Heidelberger of this College for extremely valuable advice regarding the immunological experiments. They wish to thank Mr. W. Saschek for a number of microanalyses.

SUMMARY

The preparation of the thromboplastic protein from lungs is described. Methods for the isolation of phosphatides from this lipoprotein are discussed. It was shown by means of electrophoretic experiments that, whereas at pH 5.1 a mixture of free lecithin and cephalin moved in an electric field, this was not the case with the phosphatides contained in the thromboplastic protein. Even at pH 8.8 only a very small amount of dissociation took place. The protein component, after the removal of the phosphatides, had no thromboplastic activity. The immunological properties of the thromboplastic protein, which was found to act as an antigen, are discussed.

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STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

X. THE REACTION BETWEEN HEPARIN AND THE THROMBOPLASTIC FACTOR*

BY ERWIN CHARGAFF, MORRIS ZIFF, AND SEYMOUR S. COHEN

(From the Departments of Biochemistry and Surgery, College of Physicians and Surgeons, Columbia University, New York)

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In the present communication the following findings are briefly reported. When heparin is made to react with the thromboplastic lipoprotein from lungs (1), the phosphatides are split off the protein carrier and a heparin-protein compound results. This compound, in losing its thromboplastic property, has acquired a new one: it exerts a markedly anticoagulant effect. The same series of reactions applied to a model substance, *viz.* a cephalin-histone compound (2), likewise leads to a compound containing heparin. This substance, however, which still retains considerable amounts of cephalin, is biologically inactive. This shows that the protein moiety of the thromboplastic protein, in contrast to histone or salmine, is able to enter combinations with cephalin or heparin without destruction of the biological activities of the latter substances.

There have been statements in the literature, mostly unsupported by direct experimental proof, that the anticoagulant effect of heparin was due to its reaction with thromboplastin or cephalin (3-8).[1] On the other hand, the bulk of evidence seems to point to an inhibiting action of heparin both on prothrombin and thrombin for which the presence of a component of the serum albumin fraction is required (9-12). It was found that this

* This work has been supported by a grant from the John and Mary R. Markle Foundation.

[1] To speak of a neutralization of cephalin by heparin, as has been done by several authors, is incorrect; both substances are strongly acidic.

albumin factor could not be replaced by the protein constituent of thromboplastin.

The fact that heparin under suitable conditions prevents not only the formation but also the action of thrombin shows that the reaction presented in this paper cannot be the only path by which heparin acts physiologically. However, it appears probable that both types of reaction, (*a*) the destruction of thromboplastin and (*b*) the inhibition of prothrombin and thrombin, will have to be considered for a proper explanation of the physiological effect of heparin. It may be that the presence of heparin in the site of tissue injury preponderantly leads to the first reaction mentioned, since in that case large amounts of thromboplastin are known to be liberated, whereas in the anticoagulant action of heparin in circulating or isolated blood the second reaction prevails. It should be pointed out that the reaction of heparin with the thromboplastic protein brings about the liberation of cephalin which in itself is a weak activator of clotting. The relative proportions of prothrombin, thromboplastin, and heparin present in a given system will, therefore, to a large extent decide the results of *in vitro* experiments.

It will be of interest to see whether the ability of heparin to displace acidic prosthetic groups from their protein carriers is of more general applicability.

EXPERIMENTAL

Reaction between Heparin and Thromboplastic Protein

Displacement of Phosphatides—In one set of experiments a preparation from lungs of the thromboplastic protein which had been dried in the frozen state, as described in Paper IX (1), was used. This substance contained N 12.0 per cent, P 0.87, S 0.83. Suspensions of this material in veronal buffer of pH 7.1 were shaken with solutions of heparin,[2] centrifuged, the solid residues washed with chilled water (free of CO_2), acetone, and petroleum ether, and dried *in vacuo* over P_2O_5. The composition of the reaction products is summarized in Table I. A control experiment

[2] In all experiments a preparation of the pure sodium salt of heparin was used, for which we are indebted to Hoffmann-La Roche, Inc., Nutley, New Jersey.

carried out in absence of heparin (Preparation 4, Table I) gave an interesting result. It showed that prolonged shaking of a neutral suspension of the thromboplastic protein resulted in the practically complete removal of the phospholipids, which are exceedingly difficult to extract from this protein by treatment with organic solvents (*cf.* (1)). The P content of Preparation 4 is most likely due to nucleic acid, as a comparison with data given in Paper IX (1) will show. Preparations 1 to 3, when tested with activated chicken plasma as substrate (13), showed marked anticoagulant properties. Preparation 4 had no thromboplastic activity.

As a more conclusive approach to the problem of the action of heparin on the thromboplastic protein than the one offered by the

TABLE I

Products Obtained by Shaking Thromboplastic Protein in Presence of Heparin

Preparation No.	Thromboplastic protein used	Heparin used	Total buffer volume	Duration of reaction	Reaction product	N	P	S
	mg.	*mg.*	*cc.*	*hrs.*	*mg.*	*per cent*	*per cent*	*per cent*
1	402	400	25	16	295	13.9	0.34	1.4
2	210	83	30	43	132	13.1	0.38	1.5
3	210	83	30	43	132	13.4	0.30	1.7
4	151	0	10	16	114	14.3	0.27	0.99

experiments described in the preceding paragraph, a series of experiments was carried out which were designed to demonstrate the displacement by heparin of the phosphatides contained in thromboplastin.

To 100 cc. of an approximately 1 per cent solution of the thromboplastic protein in 0.1 M borate buffer of pH 8.8 were added 300 mg. of heparin, dissolved in 10 cc. of saline. The solution was adjusted to neutrality and allowed to stand in the refrigerator for 1½ hours. It then was brought to pH 5.0 by means of 50 per cent acetic acid, when flocculation occurred. The precipitate was separated from the cloudy supernatant by centrifugation and washed three times with very dilute acetic acid of pH 5.2. All supernatants were united and examined, as will be described later. At this stage a small portion of the precipitate was emulsi-

fied in physiological saline and used for the biological tests. The bulk of the material was twice washed with acetone and dried *in vacuo* over P_2O_5 (*Preparation 5*, 800 mg. of a grayish powder). The analytical data for this and the following preparations will be found in Table II.

A parallel experiment carried out with identical quantities in exactly the same way, in which heparin was omitted, yielded *Preparation 6*, 800 mg. of a gray powder (*cf.* Table II).

This series of reactions was repeated with a different preparation of the thromboplastic protein, when *Preparation 7* (in presence of heparin) and *Preparation 8* (in absence of heparin) were obtained (see Table II).

TABLE II

Reaction between Heparin and Thromboplastic Protein in Solution

Preparation No.	N	P	S
	per cent	*per cent*	*per cent*
5	12.6	0.30	1.7
6*	12.9	0.79	0.93
7	12.1	0.29	1.8
8*	12.2	0.81	1.0

* Control experiments in absence of heparin.

The experiments here described demonstrated the displacement of the phosphatides of the thromboplastic protein by heparin. In one case the phosphatide fraction split off was isolated. It had been observed that in the experiments in which heparin was employed the supernatant solutions resulting from the precipitation of the protein at pH 5, as described above, were very cloudy, whereas the control experiments in absence of heparin yielded clear solutions. The united supernatant solutions and washings from Preparation 5 were concentrated to complete dryness *in vacuo* at 35°. The residue was extracted with boiling $CHCl_3$, and the extract filtered and evaporated. The residual oil was dissolved in a small amount of ether. On addition of acetone the phosphatides precipitated, yielding 28.5 mg. of an almost white solid. Analysis showed N 1.9 per cent, P 3.3. This substance showed slight thromboplastic activity: 0.06 mg. lowered the clotting time of 0.1 cc. of chicken plasma from 110 to 53 minutes.

The supernatant solutions from Preparation 6 (obtained in absence of heparin) treated in the same way yielded no phosphatide whatever.

Activity in Blood Coagulation—All substances were tested with chicken plasma as substrate at 30° (13). Preparations 5 and 6 were compared, as stated above, in an approximately 0.8 per cent suspension in physiological saline. When 0.03 cc. of these suspensions was added to 0.1 cc. of plasma, the clotting time of which was higher than 70 minutes, Preparation 5 prevented the clotting for more than 130 minutes, whereas Preparation 6 produced clotting in 12 minutes. Similarly, in an inhibition test with activated plasma 0.03 cc. of the suspension of Preparation 5 prolonged the clotting time of 0.1 cc. of plasma from 6 to more than 30 minutes. The supernatant obtained by centrifugation of this suspension had no anticoagulant activity.

In another inhibition test with plasma activated by the thromboplastic protein, Preparation 7 was compared with heparin. It was found to possess about 7 per cent of the anticoagulant activity of heparin. This is in satisfactory agreement with the increase in S content of Preparation 7, as compared with Preparation 8, which corresponds to a heparin content of about 5 per cent.

Heparin, as is well known, does not prevent the coagulation of fibrinogen by thrombin, unless complemented by a protein which occurs in the serum albumin fraction, but is not identical with crystalline serum albumin (9–12). It was of interest to see whether the heparin-protein compound resulting from the reaction of heparin with the thromboplastic protein could replace the albumin factor mentioned. This, as will appear from Table III, was not the case. The heparin-protein compound in itself was inactive, but the addition of serum albumin served to bring out the normal anticoagulant effect of the heparin contained in it.

The experimental results given in Table III were obtained by the method previously described (12). The substances were used in the following volumes: 0.1 cc. of a dilute thrombin solution (from human plasma) (14), 0.06 cc. of a 0.3 per cent heparin solution, 0.06 cc. of a 0.83 per cent suspension of the heparin-protein compound (Preparation 7, Table II) in saline (1.01 mg. of N per cc.), 0.06 cc. of a solution of the human serum albumin fraction insoluble at 77 per cent saturation with $(NH_4)_2SO_4$ (2.82

mg. of N per cc.). Corresponding amounts of saline were substituted for any of the factors omitted. 3 minutes later 0.2 cc. of a fibrinogen solution (prepared from human plasma) was added to the mixture. The tubes were examined for clots at fixed intervals.

Suspensions of the heparin-treated thromboplastic protein were found to split off heparin on prolonged standing, as shown by the appearance of anticoagulant activity in the supernatant solutions after centrifugation of the suspensions. It could, however, be demonstrated that the anticoagulant activity of the protein complex (Experiment 5, Table III) was not due to heparin which was merely adsorbed on the protein. The saline suspension of the

TABLE III

Influence of Heparin-Treated Thromboplastin on Clotting of Fibrinogen

Experiment No.	Components of fibrinogen clotting system*	Clotting time, min.						
		2	5	10	15	20	30	40
1	T, Tpl-He	+						
2	T, He	+						
3	T, He, Tpl-He	+						
4	T, A	−	−	−	+			
5	T, Tpl-He, A	−	−	−	−	−	−	−

\+ = clot; − = no clot.

* T = thrombin; Tpl-He = thromboplastin after treatment with heparin; He = heparin; A = albumin fraction from human serum.

heparin-protein complex used for the experiments reported in Table III was centrifuged after several days in the refrigerator, washed three times with dilute acetic acid of pH 5, and resuspended in 50 cc. of physiological saline. The activity of this suspension was compared with that of the supernatant from the last washing, the volume of which was likewise 50 cc. When tested with human plasma as substrate, clotting by thrombin took place within 1 minute in the presence of the supernatant, whereas the sample containing the heparin-protein suspension failed to clot in 2 hours.

Reaction between Heparin and Cephalin-Histone

In this experiment a cephalin-histone complex (2) which had been prepared at pH 4.7 was used. It had the following analytical

figures, N 7.9 per cent, P 2.1, S 0.40. To a suspension of 476.4 mg. of this compound in 20 cc. of veronal buffer at pH 7.2 a solution of 400.0 mg. of heparin in 5 cc. of the same buffer was added. The mixture was shaken for 16 hours. The undissolved material was separated by centrifugation, repeatedly washed with chilled water, acetone, and ether, and dried. The light brown powder weighed 302.0 mg. Analysis showed N 9.8 per cent, P 1.5, S 1.5. This substance, when tested with chicken plasma as substrate (13), showed no activity.

In another series of experiments a cephalin-histone preparation obtained at pH 7.2 was used. Analysis, N 9.4 per cent, P 1.6, S 0.40. In all cases the mixtures were shaken in veronal buffer at pH 6.9 for 24 hours and centrifuged. The solid residues were repeatedly washed with chilled water, acetone, and petroleum ether. For analysis they were dried *in vacuo* over P_2O_5 at 61°.

The reaction of 402 mg. of heparin with 501 mg. of cephalin-histone in a total volume of 26 cc. of buffer yielded 395 mg. of a light brown powder which had N 10.1 per cent, P 1.3, S 1.3. The repetition of the experiment in presence of ligroin, in which 507 mg. of cephalin-histone were shaken with 402 mg. of heparin in a total volume of 26 cc. of buffer and 10 cc. of ligroin, resulted in 417 mg. of a product which contained N 9.8 per cent, P 1.5, S 1.0. In a control experiment, in which cephalin-histone suspended in buffer was shaken with ligroin in absence of heparin, no appreciable change in the composition of the substance was observed. It was found to contain N 9.3 per cent, P 1.4, S 0.47.

The compounds which had reacted with heparin, as evidenced by the rise in S content, showed no anticoagulant activity.

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SUMMARY

The treatment of the thromboplastic protein from lungs with heparin results in the displacement of the phospholipid constituent by heparin and the formation of a heparin-protein complex with markedly anticoagulant properties. The bearing of this finding on the theory of blood coagulation is discussed. Model experiments with cephalin-histone are likewise reported.

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THE EFFECT OF ALKALOSIS ON THE CHEMICAL COMPOSITION OF BRAIN, SKELETAL MUSCLE, LIVER, AND HEART*

By HERMAN YANNET

(*From the Department of Pediatrics, Yale University School of Medicine, New Haven*)

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The data to be presented were obtained by the analysis of tissues of cats in which alkalosis was produced by a procedure designed to substitute bicarbonate for chloride without significantly altering the volume of body water. Since the concentration of serum sodium under these conditions remains relatively unchanged, the resulting alkalosis falls in the category of an alkali excess due to acid loss. From the point of view of the total extracellular electrolyte, there was probably little change in sodium accompanying loss of chloride.

Clinically, alkalosis of this type is seen principally associated with persistent vomiting in pyloric obstruction, and occasionally as a complication of alkali therapy in patients with peptic ulcer. However, in vomiting, some loss of sodium usually accompanies loss of chloride.

Previous investigations in this field have been concerned primarily with the associated metabolic and functional disorders such as renal impairment (1–6), probable change in liver function as evidenced by reduced carbohydrate tolerance, and acetonuria (7–9), and circulatory and myocardial defects suggested by reduced cardiac output (10) and changes in the electrocardiogram (11).

Procedure

Cats were used as the experimental animals. They were given the usual laboratory diet of canned salmon and milk for at least

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1 week before the procedures to be described were carried out. No food or water was offered during the experimental period. Alkalosis was produced in the following manner. A solution containing 50 gm. of glucose and 150 milliequivalents of sodium bicarbonate per liter was injected intraperitoneally in amounts approximating 100 cc. per kilo of body weight. 5 hours later, a similar quantity of fluid was withdrawn. The fluid at this time contained as an average 75 milliequivalents of chloride, 50 milliequivalents of bicarbonate, and 130 milliequivalents of sodium per liter. The animals were killed 24 hours later, by severing the great vessels at the base of the heart. All manipulations were carried out under nembutal anesthesia.

The chemical methods are given in previous publications (12, 13).

Results

The sera and tissues of twelve animals rendered alkalotic by the procedure described were analyzed. In four animals, serum was examined at the time of removal of the fluid from the peritoneal cavity and again when the animals were killed, 24 hours later. Only slight differences between the two respective sera were found. It may be assumed, therefore, that the changes found at the time the animals were killed probably were present for 24 hours. This period of time was considered adequate for equilibration to take place between tissues and blood.

The urine output during this period varied from 15 to 60 cc., averaging 40 cc., and containing an average of 100 milliequivalents of sodium and 30 milliequivalents of chloride per liter.

The clinical picture was difficult to evaluate because of the effect of nembutal which seemed to last longer than in normal animals. In two instances symptoms suggestive of tetany were observed just before the animals were killed.

Determinations of hydrogen ion concentration were carried out on seven animals in the group with alkalosis and varied from pH 7.45 to 7.80, averaging 7.60. The pH on the sera of seven normal animals varied from 7.24 to 7.40, averaging 7.35. For these determinations the method of Cullen (14) was used, as modified for the Evelyn colorimeter.

Changes in Serum

The findings in the serum have been summarized in Table I. The values for the normal animals were obtained from a previous study (15). The changes in the sera of the experimental animals consisted of an increase in bicarbonate and non-protein nitrogen and a decrease in chloride and water. While there was a greater scattering of the individual values for sodium and potassium, the average values showed little change from the normal. It will be noted that the increase in bicarbonate is only half as great as the decrease in chloride, although the concentration of total base is relatively constant. It is apparent, therefore, that a significant increase occurred in that fraction of the acids of the serum which,

TABLE I

Concentration of Serum Constituents in Normal and Alkalotic Cats

	No. of animals	H_2O	Cl	Na	Total CO_2	K	Non-protein N
		per cent	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mg. per cent*
Normal	34	94.1	119.8	152.7	20.4	5.7	31.5
		±0.3*	±0.5	±0.5	±0.6	±0.1	±2.6
Alkalosis	12	93.0	96.7	150.0	32.4	5.8	57.7
		±0.2	±2.6	±3.0	±2.0	±0.8	±4.7

* Standard error.

together with chloride and bicarbonate, makes up the total anion content. This fraction, which Gamble and Ross (16) designated as *R* in their diagrammatic representation of the acid-base balance, includes the base-binding capacity of the serum proteins, phosphate, sulfate, and organic acids. It was also found increased by Gamble and Ross in their dogs with experimental pyloric obstruction. The value for *R* in the normal group is approximately 26 milliequivalents and in the alkalotic group, 36 milliequivalents per liter of serum water. The change in pH and protein concentration in the serum increases the base-binding capacity of the proteins by 7 milliequivalents per liter. The remainder of the difference is probably related to the increases in the serum concentrations of phosphorus (4, 6, 16), sulfate (5), and ketone acids (7, 8, 17) which have been demonstrated during alkalosis.

Changes in Tissues

The results of the tissue analyses are summarized in Table II. The values are expressed per 100 gm. of fat-free solids. The last column gives the concentration of univalent base in tissue water. A difference between means less than twice the standard error of the difference is not considered significant.

Brain—There were no significant changes in the brain resulting from the experimental procedure. The finding of normal brain chloride in the presence of a marked decrease in the concentration of serum chloride should be noted.

Muscle—The decrease in muscle water of the cats with alkalosis is barely significant and explains the increase in concentration of univalent base in muscle water. The content of chloride in the muscles of the experimental cats corresponds with the value expected at the reduced concentration of serum chloride (15).

Liver—The effect of alkalosis on the composition of the liver includes an increase in water, potassium, and phosphorus and to a smaller extent, sodium. The level of liver chloride corresponds with the value expected at the reduced concentration of serum chloride (15). The increase in water therefore represents an expansion of the intracellular compartment. When compared to the increase in tissue sodium and potassium, the increase in water is only two-thirds as large as would have been required to maintain unchanged the concentration of univalent base in the tissue water. The relatively constant nitrogen levels per unit of fat-free solid indicates that there had been no significant increase in tissue glycogen, which, as Fenn (18) has shown, is deposited with water, potassium, and phosphorus. Since the volume of extracellular water, as measured by the chloride content, and the concentration of serum sodium remained unchanged, the increase in tissue sodium probably represents a slight increase in intracellular sodium.

Heart—The significant changes in cardiac muscle during alkalosis are increases in potassium and phosphorus. The chloride content is not significantly different from the value expected at the level of serum chloride (15). Since there is no change in total water, the concentration of univalent base in the tissue water is correspondingly elevated.

TABLE II

Results of Analyses of Brain, Muscle, Liver, and Heart of Normal and Alkalotic Cats

		No. of animals	Tissues, per 100 gm. fat-free solids						$\frac{Na + K}{H_2O}$
			H_2O	N	Cl	Na	K	P	
			ml.	*gm.*	*mM*	*mM*	*mM*	*mM*	*mM per l.*
Brain	Normal	11	533 ± 12*	11.0 ± 0.2	25.0 ± 0.8	37.3 ± 0.9	67.0 ± 1.8		195 ± 3.2
	Alkalosis	12	559 ± 13	11.1 ± 0.2	24.9 ± 0.9	39.3 ± 1.3	68.8 ± 1.8		193 ± 2.8
Muscle	Normal	34	345 ± 3.2	15.4 ± 0.03	5.9 ± 0.2	8.0 ± 0.2	47.4 ± 0.4	33.5 ± 0.3	160 ± 1.1
	Alkalosis	12	332 ± 5.5	15.2 ± 0.1	4.9 ± 0.3	8.4 ± 0.4	47.7 ± 0.8	33.1 ± 0.5	169 ± 2.0
Liver	Normal	13	270 ± 3.4	12.0 ± 0.3	11.2 ± 0.3	11.2 ± 0.2	30.4 ± 0.5	36.2 ± 0.6	154 ± 1.7
	Alkalosis	12	307 ± 7.6	12.7 ± 0.3	10.3 ± 0.4	12.4 ± 0.4	38.1 ± 1.4	41.7 ± 1.5	165 ± 3.0
Heart	Normal	24	410 ± 5.5	14.1 ± 0.2	19.1 ± 0.4	24.4 ± 0.9	40.1 ± 0.7	34.0 ± 0.6	159 ± 1.9
	Alkalosis	12	402 ± 7.6	14.2 ± 0.2	17.1 ± 0.8	24.1 ± 0.9	46.6 ± 1.1	36.9 ± 1.0	176 ± 3.0

* Standard error.

DISCUSSION

The changes in the chemical composition of the tissues during the alkalosis resulting from the procedure previously described might be related to three possible factors: (*a*) change in the hydrogen ion concentration within the cells, (*b*) changes in the concentration of various ions in the extracellular water, (*c*) impairment in certain physiological functions.

(*a*) A change in the hydrogen ion concentration within the cell does not appear likely for the following reasons. The increase in pH of the serum and extracellular fluids in these experiments is dependent primarily on the decrease in the concentrations of extracellular chloride, while the concentration of total base remains unchanged. The only acid that is known to diffuse freely across cellular membranes is H_2CO_3. From the average values for total carbon dioxide and pH, calculation of the CO_2 pressure shows that there has been little or no change in H_2CO_3. A few estimations of the total bicarbonate content of the various tissues in the normal and alkalotic group were carried out by the method of Danielson and Hastings (19). The actual values are not being given because the method in our hands is still not sufficiently accurate for too much significance to be placed on the absolute values. However, from a relative point of view, we have confidence, from our comparative data, in the fact that there was no evidence of an increase in tissue total bicarbonate in the alkalotic animals that could not be explained on the basis of increased concentration in the extracellular fluids. Since the pH in the cell is determined by the ratio of these two factors, *i.e.* CO_2 tension and total bicarbonate, we have reasonable evidence for believing that little if any change in hydrogen ion concentration occurred within the tissue cells of the alkalotic group.

(*b*) The changes in the content of chloride in the muscle, liver, and heart agree with data previously presented (15) describing the relationship between the concentration of chloride in the extracellular water and the tissue chloride.

The finding of a normal brain chloride at a time when serum chloride is markedly reduced is of considerable interest in view of previous work on the chloride content of brain and cerebrospinal fluid. From our previous data (20) we feel certain that an ade-

quate period of time had been allowed for equilibration between blood, cerebrospinal fluid, and brain. Presumably the procedure employed for producing alkalosis in some way influenced the interrelationship between these three factors. It has long been clear that, while cerebrospinal fluid sodium has the concentration of a serum ultrafiltrate, the concentration of chloride in the fluid is too high for such a relationship. Recently Wallace and Brodie (21) have demonstrated that chloride in the brain is in ionic equilibrium with cerebrospinal fluid rather than with a serum ultrafiltrate, as is true in other tissues like muscle, liver, and heart. Furthermore, these investigators (22) have shown that the mechanism presumably responsible for the peculiarity in the composition of spinal fluid exists not only in the ventricular chorioid plexus, but also is dependent on some function of the membranes separating plasma from the interstitial spaces of the central nervous system. It was previously demonstrated that when the body is depleted of extracellular electrolyte, the reduction in brain chloride is proportionate to the reduction in serum chloride (20). On the other hand, Amberson, Nash, Mulder, and Binns (23) found that brain chloride remained relatively constant when body chloride was replaced by sulfate. In the same experiments other tissues showed reduction in chloride proportionate to reduction in serum chloride. The depletion experiments differed from those of Amberson *et al.* and from our present alkalosis experiments in that both chloride and sodium were reduced in the serum. Consequently, it may tentatively be suggested that the concentration of chloride in the cerebrospinal fluid and the brain chloride are influenced by the concentration of serum sodium as well as by serum chloride. This hypothesis fits the recorded observations better than an assumption of large amounts of chloride fixed in some manner within the cells or extracellular structures.

Osmotic equilibrium between intracellular and extracellular fluid in the brain was shown to be obtained by loss of intracellular potassium when the concentration of serum sodium was decreased (20). In muscle, under these conditions, the major adjustment of osmotic equilibrium was by cellular hydration (15). In the present alkalosis experiments, the absence of change in brain potassium and muscle water is in keeping with these findings.

(*c*) We have no satisfactory explanation for the increase of

potassium and phosphorus in the liver and heart during alkalosis. Evidence previously cited (1–6) indicating the occurrence of renal insufficiency during alkalosis suggests the possibility that impaired renal excretion and resulting elevation of serum potassium and phosphorus may be etiologically related to the changes in the tissues. The demonstration of non-protein nitrogen retention and of the occasional increase in serum potassium and phosphorus in these studies is in line with this possibility. However, the following considerations indicate that this is probably not the case. There was no significant increase in the average concentration of serum potassium, although in four animals this was definitely elevated. There was no correlation in the individual animals between the level of serum potassium and tissue potassium. Moreover, in adrenal insufficiency (12, 24) and in nephrectomized rats (24) although a rise in concentration of non-protein nitrogen, phosphorus, and potassium in the serum can be demonstrated, there was no increase in the potassium or phosphorus contents of the liver. Furthermore, while a significant increase in muscle potassium was found in the nephrectomized and adrenalectomized animals, this was not present in the animals with alkalosis. For these reasons, the changes in serum potassium and phosphorus are probably not the direct cause of the increase in the content of potassium and phosphorus in the liver and heart during alkalosis.

While previous investigators have presented data suggesting impairment in hepatic and cardiac function during alkalosis, we have no evidence at present that would imply a causal relationship between tissue dysfunction and chemical composition.

Although the increase in liver water is probably related to the increased content of potassium, it is of interest that the resulting concentration of univalent base in the liver water is definitely elevated in spite of absence of change in the serum total base. This is also true in the heart where the increase in potassium is unassociated with any change in water. These observations are added evidence to that previously cited (15) that the concentration of univalent base in tissue water is not the sole determining factor in the maintenance of osmotic equilibrium.

Hastings and Eichelberger (25) studied the effect of alkalosis on the distribution of water in muscle. They found that when an alkaline isotonic solution is injected there is an appreciable

increase in muscle water of which a third is due to cellular swelling and the remainder due to expansion of the extracellular fluid. Our results show merely a questionable decrease in the volume of intracellular water during alkalosis. The difference in findings may be related to the fact that in our experiments there was no appreciable change in the total body water, while in those of Hastings and Eichelberger there was a retention of water equal to more than one-tenth of the weight of the animal.

It will be noticed that the urine contained an excess of sodium over chloride but that the amount excreted was so small as to have practically no effect on acid-base equilibrium. Apparently the cat does not excrete sodium rapidly enough in the presence of a marked deficit of chloride to overcome an alkalosis, in spite of the fact that total body sodium is approximately normal. This finding recalls the observation that the kidneys do not excrete water to preserve concentration in the presence of a large deficit of sodium and chloride (26). Perhaps renal adjustment does not function so as to restore normal concentration when there is a marked deficit of any of the constituents of body fluids.

SUMMARY

Cats were subjected to procedures leading to reduction in chloride, increase in bicarbonate, and no change in sodium in the serum. This resulted in a well marked alkalosis which was allowed to persist for 24 hours. The effect of this change was studied on the chemical composition of brain, skeletal and cardiac muscle, and liver.

There were no significant changes in the brain or skeletal muscle.

Cardiac muscle showed an increase in potassium and phosphorus but no change in total water or its distribution.

There were significant increases in the liver in water, potassium, phosphorus, and to a lesser extent sodium. These changes presumably represented an expansion of the intracellular compartment.

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SOME FEATURES OF THE METABOLISM OF THE CAROTENOID PIGMENTS IN THE CALIFORNIA SEA MUSSEL (MYTILUS CALIFORNIANUS)*

By BRADLEY T. SCHEER

(*From the Scripps Institution of Oceanography of the University of California, La Jolla, California*)

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One of the most striking features of marine organisms is the variety and intensity of pigmentation to be observed in many forms. Where the color is due to the presence in the tissues of chemical compounds, the possibility arises that the structure of such compounds makes them useful in the economy of the organism. The tissues of *Mytilus californianus*, where the underlying color is not masked by melanin, vary in color from very pale yellow to deep brick-red, depending on the condition of the animal, its sex, and other factors as yet undetermined. The structure of the animal is such that the pigmented tissues are not ordinarily exposed to light; it is then evident that the color, as such, can have no biological rôle, and that any importance which may be attached to the pigmentation of the tissues must perforce be physiological in nature.

Palmer (1922) was inclined to the view-point that carotenoid pigments were functionless, in the vertebrates at least; the subsequent discovery of the chemical nature of vitamin A, however, to a large extent invalidated Palmer's argument. The question now

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remains, whether carotenoids other than vitamin A and its precursors have any function in animals. The discovery that invertebrate animals are able to synthesize specific animal carotenoids (Lederer, 1938) lends credence to the view that, among certain groups of invertebrates at least, colored carotenoids are important in metabolic processes. The only metabolic studies of the carotenoids of invertebrates known to the writer have been concerned with the zoosynthesis of pigments by Crustacea (Verne, 1926; Abeloos and Fischer, 1926; Fischer 1927; Teissier, 1932; Lwoff, 1927). The synthesis of carotenoids from colorless precursors by animals has not yet been demonstrated conclusively, although the paper of Lwoff gives strong evidence for the occurrence of such a synthesis.

Mytilus was selected for a metabolic study of carotenoids from the many pigmented organisms available because earlier studies from this laboratory (Fox *et al.*, 1936, 1937) had shown its suitability for physiological work and given some information regarding its physiology. This investigation aimed to secure information with respect to the nature, origin, and metabolism of the pigments of *Mytilus*.

Methods

Preparation of Animals—The animals used were all collected from the inshore pilings of the Scripps Institution pier. The mussels were detached at low tide, care being used to avoid tearing the byssus fibers, the adhering organisms were scraped off the shells, and the mussels placed in aquaria supplied with running sea water. They were left there for at least 12 hours to permit evacuation of fecal material. For studies of the effects of fasting and of feeding various diets, five to ten animals were placed in each of a number of glass jars of about 10 liters capacity, and sea water was added; the jars were then placed in a dark room kept at a constant temperature of 20°, and the water continuously aerated by a stream of fine air bubbles. The water was changed at appropriate intervals; distilled water was added when necessary to replace loss by evaporation.

Extraction and Separation of Pigments—The volume of the entire animal (shell volume) was first determined by displacement. The

mussel was then opened by severing the posterior adductor muscle; if a quantitative estimation of pigments was desired, the animal was inverted and the water drained out of the shell. The tissues were then dissected out, weighed, and extracted by grinding with sand under acetone. The extract was concentrated by evaporation under a stream of illuminating gas at temperatures ranging from 40–60°. The pigment was now passed into petroleum ether by dilution of the acetone with water; xanthophylls were separated from esters and carotenes by partition between petroleum ether and 90 per cent methanol. The details of these procedures are discussed by Zechmeister (1934), Lederer (1938), and Strain (1938).

The total amounts of hypophasic (xanthophylls) and epiphasic (carotenes and xanthophyll esters) pigment were now estimated by spectrophotometric measurements of the absorption of light of wave-length 485 mμ by a carbon disulfide solution of the pigments. This wave-length was chosen because it was found that the region of maximum absorption of mixtures of mussel pigments always lay between 480 and 490 mμ (see Figs. 1 and 4). The values obtained by such a measurement can only be expected to be proportional to the total concentration when the relative quantities of different pigments in the mixture remain the same; however, all of the pigments obtained from the mussel were found to have absorption maxima between 475 and 500 mμ, and the absorption at 485 mμ was in no case less than 90 per cent of that at the maximum (Fig. 2). The relative values obtained for concentration of total pigment should therefore be fairly accurate. For measurements, an aliquot portion of the solution containing all of either the hypophasic or epiphasic pigments was evaporated to dryness, and the pigment residue dissolved in 5 ml. of carbon disulfide (reagent grade). The measurements were made with a Bausch and Lomb visual spectrophotometer, reading directly in density, where this quantity is defined as $d = \log_{10} I_0/I$, d being the density, and I_0 and I being the intensity of a light beam after passing through equal and measured depths of pure solvent and pigment solution respectively. In solutions which follow Beer's law, $d = ecl$, where l is the length of the absorbing path in cm., c the molar concentration of the solution, and e a constant for any particular wave-length, known as the molecular extinction

(absorption) coefficient. The measurement of density then affords a measure of the concentration if the extinction coefficient is known. The extinction coefficient of individual pigments was determined by measuring the density of solutions containing known concentrations of purified pigment. For mixtures, the extinction coefficient of crystalline β-carotene at 485 mμ was arbitrarily selected as a standard.

Individual pigments were separated by the chromatographic adsorption method of Tswett (Zechmeister and von Cholnoky, 1937). Calcium carbonate (precipitated chalk, U.S.P.) was generally used as an adsorbent, it having been found to give the best separation of mussel pigments. As solvent, petroleum ether, benzene, or ethylene dichloride was used; in most routine analyses, benzene proved most satisfactory. For elution, a mixture of 1 part of absolute ethanol with 99 parts of petroleum ether was usually successful.

Total lipids were determined by extracting the dried tissues with petroleum ether in a Soxhlet apparatus. The total lipid extract was saponified, and the organic acids separated from the non-saponifiable matter by partition between basic alcohol and ethyl ether. Organic nitrogen was determined by the Kjeldahl method on the fat-extracted tissues.

Carotenoid Pigments of the Mussel

Hypophasic Pigments—When the carbon disulfide solution of the mixed hypophasic pigments of the mussel is examined with the spectrophotometer, absorption curves similar to those shown in Fig. 1 are found. The curves shown represent most of the types obtained. It was concluded from studies of these curves that the pigments of the mussel are the same qualitatively in the two sexes, at any season, and after prolonged fasting. This conclusion is fully borne out by a study of the chromatograms obtained by passing benzene solutions of the pigments through columns of calcium carbonate. Minor differences in position and relative quantities of various pigments are seen, but, on the whole, the composition of the chromatograms is sufficiently uniform to justify the description of a typical chromatogram.

Least strongly adsorbed, and hence near the bottom of the column, is a broad yellow band (Pigment I, Fig. 2). This pigment

has been crystallized, but in too small quantities to permit of a study of all of its properties. Its spectrum, with maxima at 460,

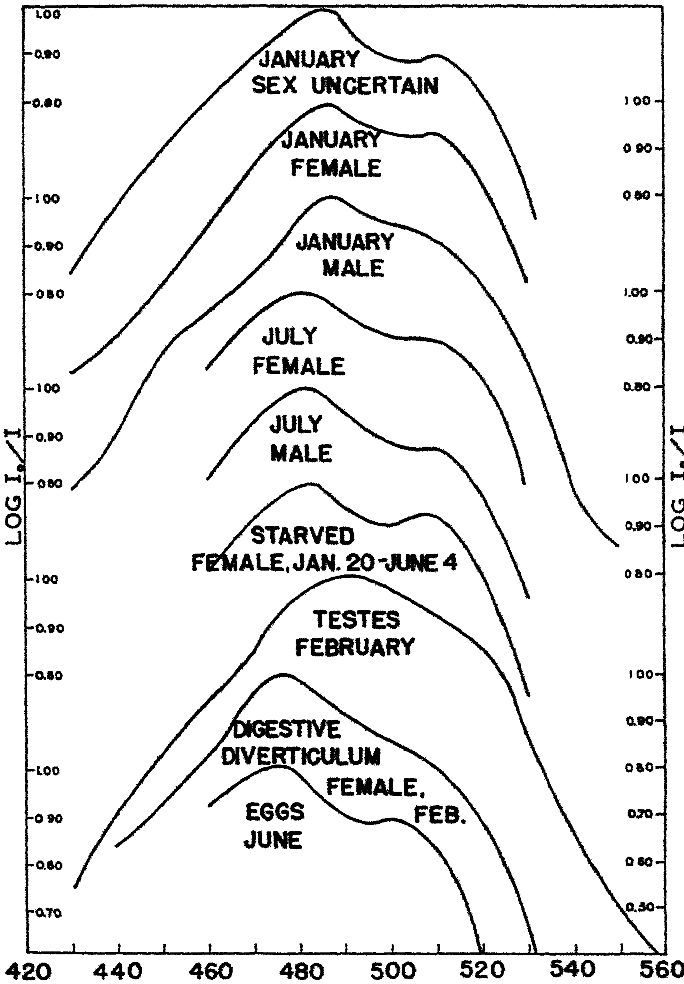

FIG. 1. Absorption spectra of hypophasic extracts of *Mytilus* tissues in carbon disulfide. Abscissae, wave-lengths in mμ; ordinates, density ($\log_{10} I_0/I$). The curves were determined by adjusting the depth of the solution so that the observed density at the maximum was equal to 1.00 (ratio of transmitted to absorbed light = I_0/I = 10). The ordinates on the left and right sides, respectively, refer to alternate curves.

482, and 513 mμ,[1] resembles those of several xanthophylls; by use of the "three tube test" (Lederer, 1938) it was proved to be identical with zeaxanthin, secured from Dr. H. Strain's laboratory through the kindness of Dr. G. Mackinney.

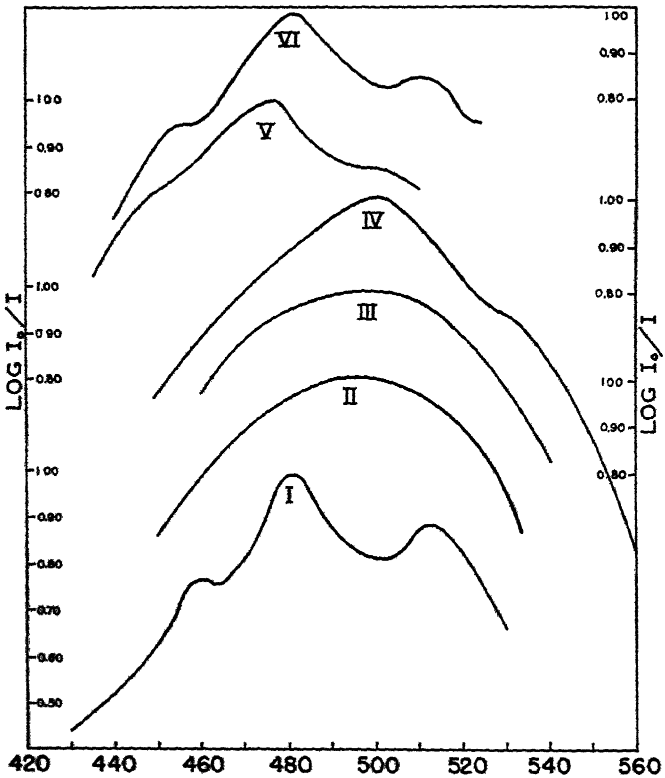

Fig. 2. Absorption spectra of individual hypophasic pigments (represented by roman numerals) from *Mytilus*. Abscissae, wave-lengths in mμ.

Above Pigment I, there always appears a thin red band (Pigment II). This pigment has not been crystallized. It lacks acidic properties, as does the similar pigment called glycymerin by Fabre

[1] Smith (1936) has studied the effect of the use of different instruments in measuring absorption spectra of carotenoids. The experimental values reported here were obtained with the visual spectrophotometer. Others are those cited by Strain (1938).

and Lederer (1934). The absorption curve of glycymerin is less symmetrical than that of Pigment II, however. Above Pigment II, there sometimes appears a thin red band (Pigment III) which is suspected of being a decomposition product of Pigment IV.

Pigment IV is present as a very broad red band, sharp below and fading to pink above. It has been obtained in crystalline state. Several hundred mussels were ground in a meat grinder and extracted with acetone. The acetone extracts were concentrated by boiling, and the pigment then taken up in petroleum ether. The hypophasic material was extracted with 90 per cent methanol, and then forced into petroleum ether by dilution with water. Some pigment separated as a flocculent precipitate at this point. The precipitate was dissolved in benzene. The petroleum ether solution was dried and evaporated, the residue likewise being dissolved in benzene. Both solutions were chromatographed. Pigment IV was separated from the other pigments, eluted, and dissolved in benzene. Addition of petroleum ether caused formation of an amorphous solid mass of pigment. This was filtered out and dissolved in methanol. Addition of water resulted in the formation of bundles of poorly defined rods (Fig. 3) decomposing at 128–131° (Berl block; corrected). Recrystallization from benzene-petroleum ether gave an amorphous precipitate decomposing at 140–144° (Berl block; corrected). Both samples were dried for several hours *in vacuo* at 60°.

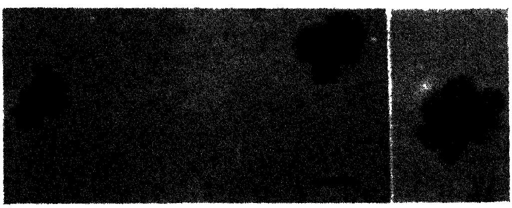

FIG. 3. Crystals of mytiloxanthin, from methanol-water. × 400

The pigment is soluble in benzene, carbon disulfide, and methanol, but only slightly soluble in petroleum ether. It is hypophasic in the partition test between 80 per cent methanol and petroleum ether, but migrates into the petroleum ether layer when the methanol is diluted to 70 per cent. The addition of base to a solution of the pigment in alcohol causes an immediate change in

color from red to yellow. Acetic acid reverses this change. When base is added to a biphasic system consisting of 70 per cent methanol, petroleum ether, and Pigment IV, the pigment forms a scarlet-red precipitate at the interface. Addition of acetic acid to the point of neutrality causes the pigment to return to petroleum ether. The pigment is resistant to treatment with alkali; after $3\frac{1}{2}$ hours at 70° in 5 per cent KOH in methanol, chromatographic comparison gave no evidence of change in composition. This pigment will be referred to in this paper as mytiloxanthin, since its properties are different from those of any carotenoid previously described. It is interesting to note that this pigment occurs in the tissues as a free acid, in contrast to astacin, which is found in many tissues as the neutral astaxanthin (Kuhn and Sörensen, 1939). It should also be noted that frequent studies of the feces, which represent a fair sample of the ingested food in view of the inefficiency of the digestive tract of the mussel, have failed to show any evidence of the presence of mytiloxanthin in the food of the mussel. Mytiloxanthin was also found to be absent from the diatom *Nitzschia closterium*, which is a constituent of the food of the mussel.

Above mytiloxanthin may occur yellow and orange bands of the type represented by Pigments V and VI. There are also found here from time to time pigments with spectra like those of Pigments III and IV. It is possible that some of these pigments are decomposition products of the main components. Pigment V, however, has a spectrum like those described for pentaxanthin (444, 474, 506 mμ) and fucoxanthin (477, 510 mμ), pigments which might be expected to occupy a similar position on the chromatogram. Pigment VI may be one of the isomers of zeaxanthin described by Zechmeister, von Cholnoky, and Polgár (1939).

Epiphasic Pigments—In Fig. 4 are presented the absorption spectra of epiphasic extracts from the same animals and tissues as the hypophasic extracts of Fig. 1. It is evident that the epiphasic spectra are as uniform as those from the hypophasic extracts, with the single exception of the extracts of the digestive diverticulum. Here, chlorophyll derivatives and other substances with strong absorption in the violet region obscure the spectra of the carotenoids. The nature of these obscuring substances has not been investigated. The chromatogram of the epiphasic extracts on

calcium carbonate is remarkably similar to that of the hypophasic extracts. Substances identical in position on the chromatogram

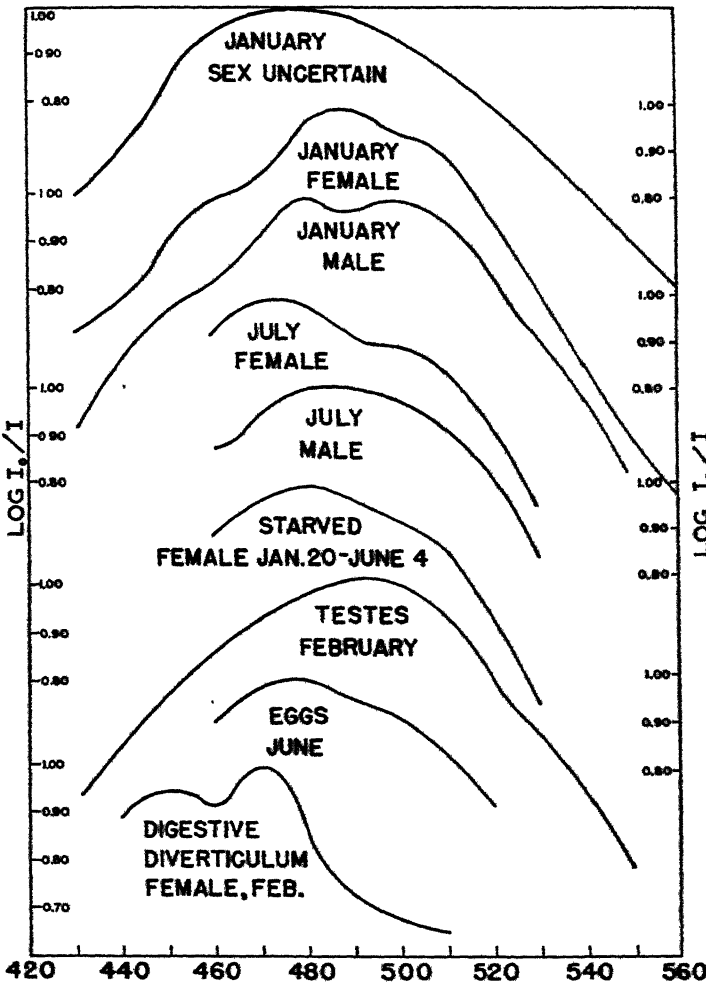

FIG. 4. Absorption spectra of epiphasic extracts of *Mytilus* tissues, in carbon disulfide. Abscissae, wave-lengths in mμ.

and in absorption spectra with Pigments I and IV have been observed; both become hypophasic after hydrolysis, and are prob-

ably esters of zeaxanthin and mytiloxanthin respectively. Two or three other bands, of small intensity and uncertain occurrence, appear from time to time. The remarkable fact, however, is that all of the pigments in the epiphasic fraction are strongly adsorbed to calcium carbonate. Very rarely, in mussels collected only a few hours before analysis, small quantities of pigments not adsorbed to calcium carbonate are observed; these are presumably carotenes. Otherwise, carotenes seem to be completely absent from the mussel, despite the fact that they are undoubtedly present in the plant material which serves as the principal food supply of this animal. Substances behaving like carotenes in respect to partition between solvents and adsorption affinities were observed in the diatom *Nitzschia closterium* and the dinoflagellate *Prorocentrum micans*, both of which (Buley, in Fox *et al.*, 1936) serve as food for the mussel, and in the feces of animals fresh from the sea.

In June, 1939, advantage was taken of the presence in the sea water off the Institution pier of large numbers of *Prorocentrum* to secure further data regarding this question. Approximately 1.5 gm. of these organisms were collected by filtering the water through a fine silk net and by centrifuging. At the same time, a sample of mussels, which had presumably been feeding on these organisms, was collected and the feces obtained. The feces, judging from the amount of light absorbed by a carbon disulfide solution of the pigments at 485 mμ, contained 4.38 mg. per cent of hypophasic pigment, while the plankton contained only 2.26 mg. per cent. However, the feces contained 0.752 mg. per cent of epiphasic pigment, while the dinoflagellates contained only 0.258 mg. per cent. That is, the ratio of hypophasic to epiphasic pigment in *Prorocentrum* was about 9:1, while the same ratio for the mussel feces was 6:1, indicating definite selection of hypophasic material in the digestive tract of the mussel.

Effects of Fasting and of Certain Other Physiological Changes on Pigments of the Mussel

Seasonal and Sexual Variations—During the winter, spring, and summer of 1939, approximately 50 normal mussels were analyzed individually for carotenoid pigments. The range of concentrations of hypophasic pigments was from 1.00 to 9.60 mg. per cent. (It should be noted that the values for concentrations and weights

given in this section of the paper are only relative, being based on determinations of the light absorbed at 485 mμ, and expressed in terms of mg. of β-carotene.) The female mussels occupied the upper portion of the range (1.76 to 9.60 mg. per cent, mean 4.95 $\pm$ 0.421, standard deviation 1.93), while the males had in general smaller concentrations of pigment (1.00 to 6.45 mg. per cent, mean 2.21 $\pm$ 1.27, standard deviation 1.32). No striking seasonal variation was apparent, although the analysis of larger numbers throughout the year might have shown some slight changes. No correlation between size and carotenoid concentration could be established.

The epiphasic pigments did not show the same relation to sex. The range obtained for males was 0.182 to 0.72 mg. per cent, with a mean value of 0.356 $\pm$ 0.03 and a standard deviation of 0.147. The values for females covered virtually the same range, from 0.169 to 0.864 mg. per cent, mean 0.34 $\pm$ 0.04, standard deviation 0.174. Again there was little evidence for any variation with season of the year or size of the animal.

Effects of Fasting—Preliminary studies were carried out in which it was attempted to alter the concentration of carotenoid by prolonged fasting. The results were insufficient to show any definite trend, except that as much as 3 months of fasting did not appear to cause any significant reduction in either epiphasic or hypophasic pigments. A carotenoid-free diet, consisting of 1 part of finely ground casein, 3 parts of starch (arrowroot or corn-starch), and 0.01 part of cholesterol was fed to certain other mussels, being suspended in the water with the mussels, and replenished as fast as removed by them. The mussels ingested this food, as was evident from the presence of abundant white feces in the jars. However, no change in total weight occurred in approximately 2 months of such feedings, during which time a control group of mussels kept in the sea at the end of the pier nearly doubled in weight. Two mussels, fasted from January 20 to May 1 and from June 23 to August 2, and fed on this diet in the intervening period, contained respectively 0.446 and 0.591 mg. per cent of epiphasic, and 3.80 and 6.29 mg. per cent of hypophasic pigments. In view of the fact that the latter animal was a male, the former being of indeterminate sex, these results would indicate that in a total period of 196 days without carotenoids no appreciable change in concentration occurred.

To test these observations carefully was subsequently attempted. Approximately 200 mussels were collected on August 17, 1939. Of these 150 were distributed among fifteen jars of sea water in a dark room at a temperature of 20°. The other 50 were analyzed as rapidly as possible. Of the fifteen experimental jars, the mussels in eight remained without food; those in another three were fed on a basal diet consisting of 1 part of dried brewers' yeast, 2 parts of casein, and 7 parts of corn-starch. The food mixture was known to yield no pigment to any of the normal carotenoid solvents. The animals in the remaining three jars were fed a pure culture of *Nitzschia closterium*. The feedings were initiated after a month of preliminary fasting, and were continued during approximately 3 months. The basal diet was usually

TABLE I

Effect of Fasting and of Various Diets on Body Weight of Mussels, Expressed by Change in Ratio of Shell Volume to Tissue Weight

Treatment	Time	Shell volume (ml.) / Tissue weight (gm.)	Shell volume (ml.) / Soma weight (gm.)	Shell volume (ml.) / Gonad weight (gm.)
	days			
Fasting	0- 30	2.50 ± 0.04	4.33	6.93
"	43- 74	3.03 ± 0.07	4.94	9.38
"	116-124	3.49	5.50	10.20
"	146-175	4.00	5.95	14.85
Nitzschia	138-169	3.60	5.64	11.12
Basal	140-160	3.92	6.09	14.45

added to the jars as rapidly as it could be removed by the mussels. About 1 liter of relatively concentrated *Nitzschia* suspension was added to each of the three jars daily. Both diets were readily ingested. The water was changed weekly in all but the jars containing the animals on the basal diet, in which it was changed daily. Feedings were usually made on 5 days of each week.

Effect on Tissue Weight—As a measure of the effectiveness of the various experimental treatments, the ratio of shell volume to wet weight of tissues was selected. The average values of this ratio for various intervals and tissues are presented in Table I. The ratio of volume to total tissue weight appears to increase linearly at a rate of approximately 0.01 per day, over the period of the

experiment. If this weight loss is assumed to result solely from the combustion of carbohydrate, a value for the oxygen consumption of the mussel at 20° of 1.25 cc. of O_2 per gm. per hour is obtained. Whedon and Sommer (1938), measuring this quantity directly, obtained values ranging from 0.3 to 2.0 cc. *Nitzschia* feeding appears to retard this weight loss, but the basal diet is without effect.

Effects of Fasting, Etc., on Individual Tissues—In order to learn whether the observed sexual differences in pigmentation were due simply to the accumulation of pigment in the ova, and to follow the movement of pigment within the body during fasting, the gonads and somatic tissues were separated in most of the analyses. From the first, it was found that the differences in concentration of hypophasic pigment were not confined to the gonads, but

TABLE II

Mean Concentration (in Mg. Per Cent) of Pigments in Gonads and Somatic Tissues of Twenty Mussels, Collected August 17, 1939, and Analyzed before September 3, with Sexual Differences and Their Standard Errors

Sex	Hypophasic pigment		Epiphasic pigment	
	Soma	Gonad	Soma	Gonad
Male	1.90 ± 0.25	5.36 ± 0.59	0.285 ± 0.08	0.691 ± 0.08
Female	4.42 ± 0.19	10.97 ± 2.3	0.413 ± 0.06	0.370 ± 0.05
Difference	2.52 ± 0.31	5.61 ± 2.4	0.128 ± 0.10	−0.321 ± 0.09

extended as well to somatic tissues. This is shown in Table II. If we choose to consider as significant only those differences which are at least twice their standard errors, we find that the hypophasic pigments of both somatic and gonad tissues and the epiphasic pigment of the gonads differ significantly in concentration between male and female tissues. The concentration of hypophasic pigment can be considered to be a true secondary sex characteristic, while that of epiphasic pigment is apparently dependent only on the accumulation of pigment in the testes, the composition being relatively uniform in all other tissues.

The results of analyses of fasted animals, and of those fed on various diets, showed no significant changes in concentration of either type of pigment in either somatic or gonad tissue. This

relative constancy of pigment concentration suggests that the disappearance of pigment proceeds at the same rate as the loss of weight by the fasting animal. Table III indicates the rate of weight loss. It is immediately evident that the weight loss is sustained mainly by the gonads, that the basal diet has no effect in retarding this loss, but that feeding *Nitzschia* serves to retard it to some extent. It should be noted that the effect of *Nitzschia* is exerted chiefly on the gonads.

Changes in Absolute Quantity of Pigment—To determine the rate at which pigment disappears, the data in Table II were used to calculate the average initial amount of pigment in a typical mussel having a shell volume of 125 ml., with tissues weighing 50 gm. The average concentration of pigment at the conclusion of the various experimental treatments was then used to determine the amount of pigment remaining in a mussel of the same shell volume, with a tissue weight corresponding to the values in Table I. The resulting quantities are presented in Table III. It will be noted that in many instances only a few animals were available for analysis. The figures must consequently be regarded as indicative only of trends.

We observe an apparent increase in amount of pigment in the ovaries of two females surviving fasting for approximately 120 days. However, this increase does not constitute proof of synthesis, in view of the fact that certain normal females had amounts of pigment slightly greater than those attained by these two animals. A high degree of retention of pigment is indicated, however. The epiphasic pigments of the ovaries of fasted females appear to increase regularly in amount with time; those females fed with *Nitzschia* have a larger amount than did the normal females at the beginning of the experiment, but somewhat less than did the females at 65 days; those fed the basal diet show a definite but small decrease in amount. It should also be noted that the largest decreases recorded in Table III are those observed in animals fed the basal diet; the ovaries, testes, and female somatic tissues especially lose large quantities of hypophasic pigment, while the testes and the somatic tissues of both sexes lose epiphasic material. In the fasted animals, the testes show the greatest and most consistent loss of hypophasic and epiphasic pigments alike. Except for the fact that the feeding of *Nitzschia* appears to retard the

TABLE III

Changes in Concentration and Total Weight of Pigment during Fasting and on Feeding Various Diets

Concentrations and weights are expressed as equivalent to mg. of β-carotene.

		Females					Males				
		Fasting			*Nitzschia*	Basal diet	Fasting			*Nitzschia*	Basal diet
Time, *days*		10	65	120	150	150	10	65	150	150	150
No. of animals		7	5	2	1	3	12	5	4	4	1
Tissue weight, *gm.*	Soma	28.8	25.3	22.8	22.2	20.6	28.8	25.3	21.0	22.2	20.6
	Gonad	18.1	13.3	12.2	11.2	8.66	18.1	13.3	8.42	11.2	8.7
Concentration of hypophasic pigment, *mg.* %	Soma	4.42	4.33	4.26	3.96	2.09	1.91	0.86	1.47	1.49	0.68
	Gonad	10.5	12.5	29.0	14.9	7.21	5.36	4.45	5.23	7.92	2.38
Weight of hypophasic pigment, *mg.*	Soma	1.27	1.20	0.970	0.880	0.430	0.551	0.216	0.309	0.331	0.140
	Gonad	1.91	1.66	3.54	1.67	0.625	0.970	0.592	0.441	0.889	0.207
Concentration of epiphasic pigment, *mg.* %	Soma	0.413	0.554	0.398	0.416	0.252	0.294	0.220	0.230	0.242	0.142
	Gonad	0.393	0.828	1.86	0.828	0.668	0.699	0.659	0.881	0.810	0.592
Weight of epiphasic pigment, *mg.*	Soma	0.119	0.140	0.091	0.093	0.052	0.085	0.051	0.048	0.054	0.029
	Gonad	0.071	0.110	0.226	0.093	0.058	0.126	0.088	0.074	0.091	0.051

loss of pigment from the tissues of males, the other changes are not of great significance.

Changes in Individual Pigments—In addition to the analysis of total pigments, the changes in concentration of zeaxanthin and mytiloxanthin were also followed. These results are summarized in Table IV. It should be noted that the values for concentration and absolute weight are here exact, being based on measurements of the absorption coefficients of pure compounds, whereas the values for total pigments were only roughly proportional to actual concentrations and weights. There appears to be a definite increase in the concentration of zeaxanthin in all tissues of fasted mussels of both sexes. Moreover, there is a definite increase in the total amount of zeaxanthin present in all tissues of other animals as well, with the single exception of the gonads of the males fed the basal diet. This increase is largest in the ovaries of fasted females, and is especially evident in the gonads of those animals fed *Nitzschia*. Mytiloxanthin, on the other hand, appears to decrease slightly in concentration. There is evidently a decrease in the absolute amount of mytiloxanthin in all but the males fed *Nitzschia*; here there is a relatively large increase in the testes, and a smaller increase in the somatic tissues. The largest decreases are observed in the gonads of the animals fed the basal diet; the somatic tissues of such animals show a decrease, as do the females fed *Nitzschia*.

Effects of Spawning on Carotenoid Content—During the course of the experiment, spawning occurred in more than half of the jars. The only consistent feature of the spawning was that it invariably occurred from 1 to 12 hours after the water had been changed; none occurred during the first 2 months of the experiment, but spawning was observed at frequent and more or less regular intervals during the succeeding 3 months. It was not possible to observe which mussels in a given jar were spawning; it was therefore necessary to assume that all mussels in a jar in which spawning was observed had spawned.

Table V shows the weights of the tissues and the amounts of pigment present in these mussels, compared with the same quantities in unspawned mussels treated similarly. Unfortunately none of the animals fed *Nitzschia* spawned. The values are all computed in the same way as those in Tables III and IV from

TABLE IV

Changes in Concentration and Weight of Zeaxanthin and Mytiloxanthin during Fasting and on Various Diets

The weights are exact, being based on absorption coefficients of the pure compounds.

		Females				Males			
		Fasting		*Nitzschia*	Basal diet	Fasting		*Nitzschia*	Basal diet
Time, *days*		65	150	150	150	65	150	150	150
No. of animals		5	2	1	3	4	4	4	1
Tissue weight, *gm.*	Soma	25.3	21.0	22.2	20.6	25.3	21.0	22.2	20.6
	Gonad	13.3	8.42	11.2	8.66	13.3	8.42	11.2	8.66
Concentration of zeaxanthin, *mg.* %	Soma	0.527	1.32	0.818	0.979	0.075	0.348	0.541	0.116
	Gonad	1.35	8.65	4.44	3.22	0.631	1.67	3.25	0.497
Weight of zeaxanthin, *mg.*	Soma	0.133	0.278	0.182	0.202	0.019	0.044	0.120	0.024
	Gonad	0.180	0.728	0.497	0.278	0.084	0.141	0.364	0.043
Concentration of mytiloxanthin, *mg.* %	Soma	2.06	1.67	1.58	1.48	0.576	0.456	0.723	0.191
	Gonad	7.92	10.98	7.92	6.40	2.90	2.87	4.88	0.710
Weight of mytiloxanthin, *mg.*	Soma	0.521	0.351	0.352	0.305	0.146	0.096	0.160	0.039
	Gonad	1.05	0.925	0.889	0.545	0.386	0.241	0.548	0.062

observed tissue weights for the spawned animals. In general it may be said that the amounts of pigment are greater in normal than in spawned fasted animals, with the reverse being true of

TABLE V

Effect of Spawning on Amount of Carotenoid (in Mg.) in Mussels Fasted and Those Fed Basal Diet

	Tissue	Fasting	Spawned	Basal diet	Spawned
		Tissue weight, gm.			
	Soma Gonad	21.0 8.42	22.0 7.03	20.6 8.66	21.9 7.63
Females. No. of animals					
		2	5	3	4
Epiphasic pigment	Soma Gonad	0.091 0.226	0.078 0.102	0.052 0.058	0.065 0.136
Hypophasic pigment	Soma Gonad	0.970 3.54	0.724 0.980	0.430 0.625	0.469 0.926
Zeaxanthin	Soma Gonad	0.278 0.728	0.210 0.287	0.202 0.278	0.141 0.274
Mytiloxanthin	Soma Gonad	0.351 0.925	0.319 0.438	0.305 0.545	0.176 0.505
Males. No. of animals					
		4	2	1	2
Epiphasic pigment	Soma Gonad	0.048 0.074	0.036 0.044	0.029 0.051	0.042 0.069
Hypophasic pigment	Soma Gonad	0.309 0.441	0.246 0.504	0.140 0.207	0.164 0.352
Zeaxanthin	Soma Gonad	0.044 0.141	0.062 0.171	0.024 0.043	0.042 0.111
Mytiloxanthin	Soma Gonad	0.096 0.241	0.103 0.195	0.039 0.062	0.053 0.129

animals fed the basal diet. The results of both carotenoid content and tissue weight measurements for spawned animals fed the basal diet are contrary to those for normal animals in that both tissue weights and carotenoid contents are generally higher in the

spawned animals. Either the two groups were not comparable at the beginning of the experiment, or these particular animals were able to make use of the basal diet, both for tissue formation and for the synthesis of carotenoids.

Aside from these results, however, it can be said that in general the amount of carotenoids present in the gonads of fasted mussels decreases subsequent to spawning. This would be expected in females, for which analysis of shed ova shows the following composition: epiphasic 0.490 mg. per cent, hypophasic 12.00, zeaxanthin 2.45, mytiloxanthin 6.50. If the difference between normal and spawned female mussels is taken to be due entirely to the shedding of eggs, however, it is found that the decrease in carotenoid content is greater than could be accounted for in this way. Furthermore, since the sperm is not pigmented, one would not expect any loss in carotenoid due to spawning in males. Nevertheless, a loss of both epiphasic pigment and mytiloxanthin has taken place from the gonads of the fasted males. It is interesting to note that the increase in zeaxanthin which has taken place in these males is almost exactly equal to the decrease in mytiloxanthin.

Effects on General Metabolism. Lipids—Table VI, the values for which were calculated in the same way as those for Table V, shows the changes in amount of total lipid, organic acid, and non-saponifiable matter during the course of the experiments. It will be noted that the total lipid shows the same type of sexual difference, when the whole animal is considered, as does the hypophasic pigment. However, when individual tissues are examined, it is found that this difference is due almost entirely to the accumulation of organic acids in the ovaries, and presumably in the eggs. The somatic tissues of the two sexes do not differ significantly from one another, and the non-saponifiable matter likewise differs little in amount in the various tissues. It will in general be seen also that the sum of organic acids plus non-saponifiable matter is never equal to the total lipid, and is often as little as half of the latter quantity. This disparity has not been explained satisfactorily.

Fasting reduces the lipid content of the mussels, but not during the first 60 days. This is in line with the view that glycogen constitutes the principal energy store of mollusks. From 40 to 50

per cent of the total lipids and from 20 to 50 per cent of the nonsaponifiable matter are lost between 60 and 150 days; about 30 per cent of the somatic organic acids of females is lost during this period, but the ovaries lose only a small fraction of their organic acids, an indication that these are deposited in the eggs, to remain isolated from the rest of the body. The males lose nearly 60 per cent of their testicular organic acids during fasting. The nonsaponifiable matter shows the same trend, being lost in considerable quantities from all tissues of both sexes. The feeding of *Nitzschia* retards this loss of lipids from males. The feeding of the basal diet is virtually without effect in females.

Table VI

Changes in Lipids of Mussel during Fasting, on Feeding Various Diets, and after Spawning

	Treatment	Days	No. of animals	Total lipid		Organic acids		Non-saponifiable matter	
				Soma	Gonad	Soma	Gonad	Soma	Gonad
				mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Males	Fasting	10	6	254	232				
	"	60	8	224	226	60.5	84.4	61.0	57.0
	"	150	3	141	107	37.8	35.0	33.0	30.6
	" spawned	150	3	163	94.9	47.4	41.3	41.9	26.8
	Nitzschia	150	5	154	150	37.4	49.6	41.3	36.1
	Basal diet	150	0						
	" " spawned	150	2	131	82.0	36.8	23.2	32.9	23.1
Females	Fasting	10	3	294	532				
	"	60	5	295	425	77.6	142	53.1	75.5
	"	150	4	156	264	56.7	137	41.4	46.0
	" spawned	150	4	182	154	60.0	65.5	40.0	26.8
	Nitzschia	150	1	199	346				
	Basal diet	150	5	154	185	43.5	99.4	32.0	38.0
	" " spawned	150	4	134	86.5	26.9	31.0	30.2	18.4

Water, Protein, Carbohydrate—The water content of the tissues remains nearly constant throughout, the mean values for the somatic tissues being 83.00 ± 0.363 per cent and for the gonads 81.65 ± 0.834 per cent; the difference between the two types of tissue is not significant. The values are lower than those obtained by Dr. D. L. Fox (personal communication) on whole mussels,

owing to an inevitable loss of blood during dissection. The protein content of the tissues, expressed as 6.25 times the organic nitrogen content of the fat-extracted tissues, shows no difference with sex, and is only slightly higher in the gonads (10.6 per cent) than in the somatic tissues (9.8 per cent) after 60 days of fasting. Further fasting results in only a slight decrease in the somatic tissues though a noticeable change takes place in the gonads. In fasted animals, there is a tendency for the protein concentration to increase, although a few individuals show a marked decrease. In animals fed *Nitzschia* and the basal diet, the concentration falls sharply below the level of the fasted animals. This is likewise true in general of spawned animals, the gonads of which have very low protein concentration, regardless of sex.

Since the fat content is small compared with that of proteins, it may be assumed that carbohydrate concentration varies inversely with that of proteins. If this is true, then we may say that carbohydrates decrease in concentration in the gonads of fasted animals between the 2nd and 6th months of fasting. In the somatic tissues, there is little change in concentration; in the gonads of animals fed *Nitzschia* and the basal diet, the decrease is retarded. It appears likely also that in fasted mussels the concentration of carbohydrates in the gonads of spawned is larger than that in unspawned mussels.

DISCUSSION

Zechmeister (1937) divides vertebrates into four groups, with respect to the types of carotenoid stored; the mussel is evidently similar to animals such as the birds in that only xanthophylls are selected from the dietary supply of carotenoids available. Furthermore, there is evidence, from the relative simplicity and constancy of the qualitative picture presented by the carotenoids of the mussel, that only a few xanthophylls are selected.

These few pigments are subjected to certain transformations by the mussel, including esterification and the synthesis of the animal pigment mytiloxanthin. Some light is thrown on this synthesis by the experiments during fasting; in the fasted males, the amount of mytiloxanthin showed a progressive decrease. When the animals were fed the diatom *Nitzschia*, known to contain no mytiloxanthin, the amount of mytiloxanthin, instead of decreas-

ing, increased. Evidently some substance present in *Nitzschia* serves as a precursor for mytiloxanthin.

Further evidence of synthesis of pigment by the mussel is provided by the fact that zeaxanthin increases in amount during fasting; such an increase takes place when mussels are fed on the basal diet as well. When *Nitzschia* is fed, furthermore, zeaxanthin accumulates to a much greater extent than during fasting. Certain observations of Heilbron and Phipers (1935) are of interest in this connection; on the basis of rather scanty evidence, they postulate a conversion of fucoxanthin to zeaxanthin in the alga *Fucus vesiculosus*. Strain (1938) finds no evidence of such a change in higher plants, but, in view (1) of the presence of a substance (Pigment V, Fig. 2) resembling fucoxanthin in the mussel and in *Nitzschia*, and (2) of the report of Heilbron and Phipers that a substance resembling mytiloxanthin in some of its properties is found in *Fucus*, further chemical and physiological study of the interrelations of fucoxanthin, mytiloxanthin, and zeaxanthin should be of interest.

The most striking quantitative feature of the pigmentation of the mussel is its variation with sex. As was noted in the experimental part, both somatic and gonad tissues of females contain a higher concentration of hypophasic pigment than similar tissues of males. The association of carotenoids with sex is a common one. Most ova of marine animals are colored by carotenoids, and the corpus luteum of mammals acquired its name from the color conferred upon it by its carotenoid content. The work of Moewus (1939) indicates that the pigments crocin and crocetin are concerned with the development of motility and with conjugation in gametes of the alga *Chlamydomonas*. Sexual differences in pigment concentration are fairly common in animals (Verne, 1926) and are known in plants (Emerson and Fox, 1940).

The results of the fasting experiments permit several conclusions. First of all, it is noted that the metabolism of carotenoids is apparently not an intense one. The pigments disappear during fasting at a rate which insures that their concentration in the tissues rarely falls by any significant amount, and may even increase slightly. It appears that loss of pigment is accelerated by two factors. First, those animals fed the basal diet appear to have lost more pigment than the fasted animals. Secondly,

spawning seems to result in a loss far in excess of that to be expected from the mere removal of carotenoid in the eggs. If we assume, on the basis of most of the determinations of body weight, that the basal diet was not utilized by the mussels, while some benefit was derived from the *Nitzschia* cells which were fed, we may derive a tentative explanation of these phenomena. The feeding process, in the mussel, involves (1) production of a current of water through the mantle cavity by means of the ctenidial cilia, (2) secretion of mucus by the ctenidial cells, (3) entanglement of food particles in the mucus, with transfer of the mucus strands containing food to the mouth by the action of the cilia. Fasting mussels, living in water completely free of suspended matter, would presumably not carry on this process to any considerable extent; only a current of water sufficient for respiratory purposes would probably be produced. Feeding mussels, on the other hand, would be active continually in filtering the water and ingesting food. If they were unable to digest the basal diet, they would have no source of material to replace the substances consumed by the activities of feeding; on the other hand, by digesting and absorbing a portion of the *Nitzschia* material, they would be able to replenish these substances to some extent. It seems likely, then, that tissue carotenoids are consumed in the course of the activities associated with feeding. The association, in the vertebrates, of vitamin A with the integrity of mucous surfaces is brought immediately to mind.

The disappearance of carotenoids from the gonads of fasted animals may be taken as an indication that the carotenoids play some rôle in gametogenesis. This view-point is strengthened by the observation that the gonads act as a store of reserve carotenoids, drawn upon in fasting. The gonads also serve as a store of carbohydrate, but not of lipid, which disappears uniformly from all the tissues in fasting. In line with this last observation, it should be noted that the distribution of carotenoids is independent of that of other lipids; this is contrary to the assertion which is sometimes made (see Zechmeister (1937)) that the distribution of carotenoids in the animal body is dependent largely on their solubility in fatty substances. Both lipids and carotenoids, however, are accumulated to a considerable extent by the eggs.

SUMMARY

1. The results of a qualitative study of the carotenoid pigments of *Mytilus californianus* are reported.

2. It is found that only xanthophylls of a few types and their esters are present in the tissues of the mussel; *Mytilus* selects the xanthophylls of the food and rejects the carotenes.

3. A new acidic xanthophyll with an absorption maximum at 500 mμ is described, and provisionally referred to as mytiloxanthin.

4. Quantitative studies of the distribution of carotenoids in *Mytilus* show that the concentration of hypophasic pigments in all tissues of female mussels is higher than that of males.

5. The changes in concentration and amount of carotenoid, lipids, organic nitrogen, and water were followed during a period of 6 months, in mussels subsisting on a basal carotenoid-free diet, on a diet of *Nitzschia closterium*, and without any source of food.

6. It is found that the gonads act as a store of carotenoid and of carbohydrate, but not of lipid. The concentration of carotenoids is independent of that of lipids.

7. Prolonged fasting results in a very small loss of pigment; during fasting mytiloxanthin disappears and zeaxanthin is formed.

8. Both mytiloxanthin and zeaxanthin increase in amount when the diatom *Nitzschia* is fed; mytiloxanthin is not present in *Nitzschia*.

9. The activities of feeding and of gametogenesis are shown to be associated with an increased destruction of pigment.

10. The results of the investigation are definitely in favor of the view that carotenoids play a positive rôle in the metabolism of *Mytilus*.

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THE UTILIZATION OF CARBON DIOXIDE IN THE SYNTHESIS OF α-KETOGLUTARIC ACID*

Sirs:

The utilization of carbon dioxide in photosynthesis and in the metabolic processes of certain bacteria,[1] plants,[2] and yeast[3] has been clearly demonstrated. Using radioactive carbon, we have been able to show a comparable assimilation of carbon dioxide by the tissues of a higher animal; namely, in the synthesis of α-ketoglutaric acid from pyruvic acid by pigeon liver.

The protocol of a typical experiment follows. 8.1 gm. of minced pigeon liver were suspended in 60 ml. of the Krebs saline-bicarbonate medium,[4] pH 7.4, in which the bicarbonate had been prepared from radioactive carbon (C_{11}) obtained by the bombardment of boron with deuterons. 1.7 ml. of 0.1 M sodium malonate were added and the tissue suspension equilibrated with a mixture of 95 per cent O_2 and 5 per cent CO_2 for 10 minutes at 40°. 10 ml. of 0.2 M sodium pyruvate were added and the reaction vessel shaken vigorously for 40 minutes at 40°. The solution was deproteinized with metaphosphoric acid and, after the addition of a small quantity of α-ketoglutaric acid to act as a carrier, the α-ketoglutarate was precipitated as the 2,4-dinitrophenylhydrazone. The precipitate, after thorough washing with 10 per cent HCl and drying *in vacuo*, melted at 222° (uncorrected)

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and showed no depression of the melting point on being mixed with a sample of the hydrazone. The substance was intensely radioactive (as measured by a Lauritzen electroscope) and contained about 3 per cent of the added activity. The hydrazone was recrystallized from 50 per cent ethanol, washed, dried *in vacuo*, the melting point measured (222°, no depression with the control sample), and the activity determined. The activity of the recrystallized material compared with the original precipitate corresponded closely to that calculated on the basis of the rate of decay of the radioactive carbon (21 minutes) and the time taken for recrystallization, indicating the identity of the radioactivity with α-ketoglutaric acid.

The data are unequivocal in demonstrating the participation of carbon dioxide in the synthesis of α-ketoglutaric acid from pyruvic acid in the pigeon liver. Since pigeon liver can synthesize α-ketoglutaric acid from oxaloacetic acid plus pyruvic acid,[5] the data suggest, as the simplest hypothesis, that carbon dioxide combines directly with pyruvic acid to yield oxaloacetic acid, the latter then combining with an additional molecule of pyruvate to form α-ketoglutarate by the reactions previously described.

Department of Biochemistry
University of Chicago
Chicago
E. A. Evans, Jr.

Department of Physics
University of Chicago
Chicago
Louis Slotin

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DELPHININE

III. THE ACTION OF HYDROCHLORIC, NITRIC, AND NITROUS ACIDS ON DELPHININE AND ITS DERIVATIVES

By WALTER A. JACOBS AND LYMAN C. CRAIG

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

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In further attempts to find suitable means for the controlled degradation of the aconitine molecule complications have been encountered paralleling in part some of the experience of previous workers and which have made difficult more rapid progress. It was hoped by certain methods which will be described in another connection to remove the hydroxyl groups and to reach a more suitable starting point for further degradation. But because of the complexity of the reaction mixture obtained as a rule, it became desirable to select a simpler alkaloid of the group. Attention was therefore turned to delphinine because of its fewer hydroxyl groups. This work has established the formula $C_{33}H_{45}O_9N$ and has made conclusive its relationship to the aconitine group of alkaloids (1). As in the case of the long known reaction with aconitine, delphinine when heated in methyl alcoholic solution readily suffers replacement of its acetoxyl group by methoxyl, with the production of *methylbenzoyldelphonine*. (*Delphonine* is the alkamine.)

Systematic attempts have been made to remove the free OH group of delphinine or certain of its derivatives or to replace it by halogen for subsequent removal. But in such experiments complications continued to crop up which required individual study at each step. Thus a curious contrast has appeared in the behavior of delphinine itself and its oxidation product α-oxodelphinine. The latter, unlike the alkaloid, was unaffected on heating under comparable conditions (or even at 130–140°) with methyl alcohol alone. However, if, as previously reported (1), the sol-

vent contained HCl (3 per cent), replacement of the acetoxyl group by methoxyl occurred, with formation of *methylbenzoyl-α-oxodelphonine*, $C_{32}H_{43}O_9N$. At the time, the production was also recorded of an amorphous basic fraction which has since been found to be a mixture. On treatment with nitrous acid a neutral *nitroso derivative* has been obtained from it, analysis of which approached a formula $C_{32}H_{42}O_{10}N_2$. It is possibly a derivative of a secondary amine isomeric with methylbenzoyl-α-oxodelphonine and formed from the latter by the isomerizing action of methyl alcoholic HCl.

The above methylbenzoyl-α-oxodelphonine has now also been obtained in good yield by oxidation of methylbenzoyldelphonine with permanganate in acetone solution. The simultaneous production of an isomeric β-oxo derivative such as occurs in the oxidation of the alkaloid itself, however, could not be detected.

During these reactions with delphinine or its α-oxo derivative there was thus no indication of cleavage of the OH group to form unsaturated derivatives. The action of HCl was then studied under more rigorous conditions. Although the alkaloid itself was apparently little affected under such conditions, when α-oxodelphinine was treated at 25° with methyl alcohol saturated with HCl at 0° a succession of reactions occurred. After about 24 hours a chloro derivative was isolated in good yield, analysis of which indicated a formula $C_{32}H_{40}O_9NCl$ in which one methoxyl group has been replaced by Cl. If the reaction was allowed to proceed longer (a week), further change occurred. From the resulting mixture a substance was obtained analysis of which approximated a formula $C_{32}H_{39}O_8NCl_2$ in which the hydroxyl group appears to have been replaced by a 2nd Cl atom.

In the case of the above monochloro derivative preliminary attempts were made to replace the chlorine by hydrogen. On hydrogenation with platinum oxide catalyst, however, only the benzoyl group was reduced with production of a *hexahydrobenzoyl derivative*, $C_{32}H_{46}O_9NCl$.

When the chloro derivative was heated with methyl alcohol at 100°, the Cl atom was removed but a complicated mixture of neutral and basic substances resulted, the interpretation of which has proved very difficult and is only in a preliminary stage. Several possibly isomeric neutral substances were isolated of doubt-

ful homogeneity, analysis of which suggested a formula $C_{32}H_{39}O_9N$ resulting from cleavage of HCl from the chloro compound. From the basic fraction a crystalline basic substance was obtained, also apparently a mixture, analysis of which approximated the same formula $C_{32}H_{39}O_9N$. The formation of a neutral *nitroso derivative* from the latter indicated it to be a secondary amine. However, the methoxyl determinations of all of these substances have not given results in accord with the required values for either two or three OCH_3 groups. Although therefore the homogeneity is doubtful, it is nevertheless important to place on record our observations awaiting their final interpretation. This experience demonstrates that many competing reactions can participate under such simple conditions, a fact which has contributed much to the complexity of the problem. An attempt was made to simplify the problem by making use of methylbenzoyl-α-oxodelphonine in order to avoid the complication of partial replacement of the acetoxyl group by methoxyl during these reactions. But the reaction between this substance and saturated methyl alcoholic HCl was found to present in itself a complicated picture.

As in the case of α-oxodelphinine, when β-oxodelphinine was heated with methyl alcoholic HCl (4 per cent) replacement of the acetoxyl group by methoxyl occurred with the production of *methylbenzoyl-β-oxodelphonine* isomeric with the previously mentioned α derivative. However, here the analogy ended, since unlike the α isomer it was largely recovered unchanged after exposure to the action at room temperature of methyl alcohol saturated with HCl. Similarly, methylbenzoyl-β-oxodelphonine was largely unaffected by this reagent. The greater apparent resistance in the β derivative of the methoxyl group which is more labile in α-oxodelphinine was again brought out in the contrast in the behavior of each of these isomers towards nitric acid. The β derivative appeared to suffer little change when its solution in HNO_3 (1.42) was allowed to stand at 25° for several days. In the case of α-oxodelphinine, however, demethylation of one methoxyl group occurred with the production of *the substance* $C_{33}H_{41}O_{16}N$. But substitution of the methoxyl group by a nitro group, as might have been expected from the results with strong HCl, did not occur.

The action of these reagents on pyro-α-oxodelphinine was similarly studied. Here a curious behavior was noted. When dissolved in aqueous HCl (1.19), it was gradually changed to a *chloro derivative* with replacement of one methoxyl group; *viz.*, $C_{30}H_{36}O_7NCl$. If methyl alcohol saturated at 0° with HCl was employed, two methoxyl groups were replaced by 2 chlorine atoms, since analysis of the resulting crystalline *dichloro derivative* agreed with the formula, $C_{29}H_{33}O_6NCl_2$. When the reaction was continued for a week, a mixture resulted but only 50 per cent of crystalline material could be recovered, analysis of which still approximated the figures of the above dichloro derivative. When the latter was heated with methyl alcohol, Cl was again replaced by methoxyl groups. The resulting substance proved to be isomeric with pyro-α-oxodelphinine and apparently identical with the substance previously obtained from it by heating with 4 per cent methyl alcoholic HCl (1). In the attempt to replace halogen by hydrogen the dichloro derivative on hydrogenation yielded a *hexahydro derivative*, $C_{29}H_{39}O_6NCl_2$, in which only the benzoyl group was reduced.

Finally, a preliminary study of the action of nitric acid on pyro-α-oxodelphinine has been made. In this case the concentrated acid caused rapid removal of one methoxyl group with the production of a *monodesmethyl derivative*, $C_{30}H_{37}O_8N$. If allowed to act longer or at a higher temperature, however, the reaction became more involved. When the substance $C_{30}H_{37}O_8N$ was treated with HCl (1.19), it was transformed into a *monochloro derivative*, $C_{30}H_{36}O_7NCl$, apparently isomeric with the substance obtained directly under the same conditions from pyro-α-oxodelphinine itself.

A study of the action of hot dilute nitrous acid upon delphinine has proved of interest. From the reaction mixture about 10 per cent of a sparingly soluble neutral *nitroso derivative* was obtained, analysis of which agreed with the formula $C_{33}H_{44}O_{10}N_2$. Although this substance still retained four methoxyl groups in addition to the original acetyl and benzoyl groups, the $N(CH_3)$ determination gave but a small fraction of the amount exhibited by the original alkaloid. The question whether this is a nitroso derivative of a secondary amine produced by isomerization of delphinine during the reaction is now the subject of further study. Its production is in all likelihood in part analogous to that of the nitroso compound

obtained under similar conditions from aconitine and for which Lawson (2) derived an incorrect formula $C_{31}H_{40}O_{12}N_2$. The latter will be discussed in another connection.

The major portion of the reaction mixture proved to be of basic character and a crystalline base was obtained from it. Analysis of the latter agreed with the formulation $C_{33}H_{45}O_{10}N$; *i.e.*, of a *hydroxydelphinine*. The methoxyl and $N(CH_3)$ determinations were consistent with this interpretation. The new hydroxyl group appeared to be of secondary character and attached to a carbon atom vicinal to the N atom, since on oxidation in acetone solution with permanganate under conditions paralleling the production of the oxodelphinines from delphinine, the new base also yielded a neutral oxodelphinine. The hydroxyl group must therefore be the point of oxidation to CO. The new oxidation product which is provisionally called *γ-oxodelphinine* was superficially indistinguishable from β-oxodelphinine except in rotation. That of the former in acetic acid was $[\alpha]_D^{20} = +40°$ as against $[\alpha]_D^{20} = +31°$ of the latter. However, when it was heated with methyl alcoholic HCl, a pentamethoxyl derivative was obtained, *viz. methylbenzoyl-γ-oxodelphonine* ($[\alpha]_D^{30} = +5°$ in methanol), which showed a still greater difference in rotation from that of the methylbenzoyl-β-oxodelphonine recorded above ($[\alpha]_D^{30} = +27°$ in methanol). Its further study has been deferred for the present.

In a previous paper (1) the suggestion was offered that the pyrolytic production of pyrodelphinine from delphinine with loss of acetic acid is probably accompanied by the formation of an oxidic bridge, a conclusion drawn by Schulze and Liebner (3) in the case of pyraconitine and pyraconine on the basis of acetylation studies. This has more recently been supported by the published acyl determinations of Tamura (4) on the so called diacetylpyromesaconitine and triacetylpyroxonine. However, such an interpretation was discarded by Sharp (5) on the basis of his study of pseudoaconitine. Along with other confirmatory observations he found that the fully acylated alkamine which contains no free OH group, *viz.* tetraacetylpseudoaconine, still loses acetic acid on pyrolysis with production of triacetylpyropseudoaconine. Because of the failure to hydrogenate, Sharp concluded that a new carbon to carbon link was produced by the reaction.

We have already similarly reported (1) that pyrooxodelphinine

yields a hexahydropyrooxodelphinine in which only the benzoyl group is hydrogenated. The failure to hydrogenate, however, does not eliminate the possibility of the presence of a resistant double bond formed in the reaction.

With the hope of obtaining direct evidence of the non-participation of a free OH group in the formation of pyrodelphinine acylation of the latter with acetic anhydride was studied. Although such a reaction appeared to occur no crystalline product could be isolated. In the case of pyro-α-oxodelphinine acylation was useless, since α-oxodelphinine itself did not react directly with acetyl chloride. It was also recovered unchanged on attempted benzoylation in pyridine. The benzoyl derivative was obtained, however, indirectly. Delphinine when benzoylated in pyridine solution yielded *benzoyldelphinine* which on oxidation with $KMnO_4$ was converted into *benzoyloxodelphinine.* Both of these benzoyl derivatives although containing no free OH groups continued to lose acetic acid on heating. Unfortunately, the resulting compounds could not be obtained in crystalline form. For the present, the general nature of the pyrolytic transformation of these alkaloids and their derivatives in terms of previous proposals must be regarded as uncertain.

EXPERIMENTAL

Methylbenzoyldelphonine—5 gm. of delphinine in 75 cc. of methyl alcohol were heated at 100° for 20 hours. Cleavage of acetic acid was apparent. After partial concentration and careful dilution with water and dilute Na_2CO_3 a colorless resinous precipitate formed which rapidly crystallized. The collected material weighed 4.3 gm.

After recrystallization from dilute methyl alcohol it formed small, short, often pointed prisms which melted at 173–175°.

$$[\alpha]_D^{25} = +27° \ (c = 1.72 \text{ in } 95\% \text{ alcohol})$$

$C_{32}H_{45}O_8N$.	Calculated.	C 67.21, H 7.94, OCH_3 27.16
	Found.	" 67.56, " 7.91, " 26.82

Delphinine and Methyl Alcoholic Hydrochloric Acid—0.12 gm. of delphinine in 2 cc. of absolute methyl alcohol containing 3.3 per cent of HCl was heated at 100° for 18 hours. After concentration copious crystallization of the hydrochloride which melted at 213–214° occurred from methyl alcohol and ether.

$C_{33}H_{45}O_9N \cdot HCl$.	Calculated.	C 62.28, H 7.29, OCH_3 19.52
	Found.	" 61.70, " 7.22, " 19.20

Similarly, 0.23 gm. of the alkaloid was dissolved in 4 cc. of methyl alcohol saturated at 0° with HCl and allowed to stand at room temperature for 45 hours. The alkaloid was recovered from this solution in excellent yield and possessed the properties of delphinine.

$C_{33}H_{45}O_9N$.	Calculated.	C 66.07, H 7.57
	Found.	" 66.09, " 7.75

Methylbenzoyl-α-Oxodelphonine—A solution of 4.3 gm. of methylbenzoyldelphonine in 450 cc. of dry acetone containing 4.5 cc. of acetic acid was treated with 0.45 gm. of potassium permanganate. Oxidation gradually occurred in the mixture which was held at about 25° and by next morning the reagent was used up. After concentration and dilution copious crystallization occurred, and on further dilution of the mother liquor with final concentration, additional fractions were obtained. The total yield was 3.9 gm. No appreciable differences in melting points or rotations were noted in the different fractions. The substance formed lustrous platelets from dilute methyl alcohol which on rapid heating melted at 221–223° and then resolidified and melted again at 236–237°.

$$[\alpha]_D^{25} = -41.5° \ (c = 1.03 \text{ in methyl alcohol})$$

$C_{32}H_{43}O_9N$.	Calculated.	C 65.60, H 7.40, OCH_3 26.47
	Found.	" 65.70, " 7.33, " 26.33

This oxidation product proved to be identical in properties with the substance obtained previously (1) by the action of methyl alcoholic hydrochloric acid on α-oxodelphinine and showed no melting point depression when mixed with it. A recent determination of the rotation of the earlier substance has been found to be $[\alpha]_D^{25} = -35.5°$ ($c = 0.99$ in methyl alcohol). The low methoxyl values reported with this material indicated some contamination.

α-Oxodelphinine and HCl—The action of hot 3 per cent absolute methyl alcoholic HCl as previously (1) described gave in addition to the neutral methylbenzoyl-α-oxodelphonine an amorphous basic fraction. This material which was of doubtful homo-

geneity has since been found to give in very small yield the following nitroso derivative.

A solution of 60 mg. of the amorphous base in 8 cc. of water containing 0.5 cc. of acetic acid was treated at room temperature with 0.2 cc. of 30 per cent sodium nitrite solution. A flocculent precipitate gradually formed and on standing overnight this was found to be partly crystalline. After collection and drying it was dissolved in chloroform and concentrated and then treated with ether. On further concentration to obtain a proper balance of the two solvents, it gradually crystallized as minute four cornered tablets which melted slowly with decomposition at 228–230° after preliminary softening.

It gave the typical nitrosamine reaction with phenol and H_2SO_4.

$C_{32}H_{42}O_{10}N_2$.	Calculated.		C 62.50, H 6.89, N 4.56
	Found.	(a)	" 62.67, " 6.75
	"	(b)	" 62.05, " 6.88, N 4.65

The ineffectiveness of methyl alcohol without HCl was shown as follows: 0.25 gm. of α-oxodelphinine was heated with 10 cc. of absolute methyl alcohol at 130–140° for 17 hours. On concentration to 4 cc., copious crystallization of starting material occurred.

$C_{33}H_{43}O_{10}N$.	Calculated.	C 64.56, N 7.07
	Found.	" 64.54, " 6.89

The Substance $C_{32}H_{40}O_9NCl$—2 gm. of α-oxodelphinine were placed in a sealed tube with 35 cc. of methyl alcoholic HCl which had been saturated at 0°. After agitation at room temperature for almost 30 minutes the suspension gradually dissolved and the solution was allowed to stand at about 25° for 21 hours. The clear solution was carefully concentrated *in vacuo* to dryness and the residue was dissolved in chloroform. The latter was shaken with a little Na_2CO_3 solution to remove HCl and then repeatedly extracted with 2 per cent H_2SO_4 to dissolve any basic material which proved to be negligible in amount. The remaining neutral chloroform solution was washed and dried. On concentration to about 5 cc. and treatment with ether, a copious globular crystalline powder separated which was collected with ether. The yield was 1.44 gm. This was recrystallized by solution in a good volume of hot acetone and concentration to 25 cc. On standing,

delicate needles gradually separated which after preliminary sintering melted at 242–243° with effervescence.

$$[\alpha]_D^{25} = -60° \ (c = 1.17 \text{ in chloroform})$$

For analysis the substance was dried at 120° and under low pressure.

$C_{32}H_{40}O_9NCl$.	Calculated.	C 62.16,	H 6.53,	Cl 5.74,	OCH_3	15.06
	Found. (a)	" 62.85,	" 6.68,	" 4.99,	"	14.85
	" (b)	" 62.54,	" 6.83,		"	15.26

14.330 mg. of substance were refluxed in 3 cc. of 0.1 N NaOH and 3 cc. of alcohol for 3 hours and titrated back against phenolphthalein. Found, 0.716 cc.; calculated for 3 equivalents, 0.696 cc.

The purification of this substance was complicated somewhat by the fact that when the reagent was allowed to act longer increasing amounts of a dichloro derivative were produced, as shown below. At the same time, on recrystallization from chloroform mixtures, retention of solvent occasionally occurred, so that the Cl determinations proved to be misleading.

For instance material contained in the mother liquor obtained in the above recrystallization was recovered by addition of ether to the concentrated chloroform solution.

On analysis of the substance C 61.41, H 6.44, Cl 8.60 were found.

However, when this was dissolved in acetone and concentrated, two successive fractions were obtained to the extent of at least 60 per cent. The first showed $[\alpha]_D^{25} = -55°$ and the second $[\alpha]_D^{25} = -57°$.

Found, (a) C 62.55, H 6.35, Cl 5.70; (b) Cl 6.07

Hydrogenation of $C_{32}H_{40}O_9NCl$—0.1 gm. of the chloro derivative was hydrogenated in acetic acid solution with 50 mg. of the platinum oxide catalyst of Adams and Shriner under a pressure of 3 atmospheres. The reaction was complete in 35 minutes after absorption of 3 to 4 moles of H_2 in excess of the catalyst requirements. The hexahydro derivative formed needles from methyl alcohol which melted at 229° with slow effervescence, and gave a strong halogen test.

$C_{32}H_{46}O_9NCl$.	Calculated.	C 61.55,	H 7.43
	Found.	" 62.03,	" 7.44

On saponification the characteristic odor of hexahydrobenzoic acid was obtained.

The Substance $C_{32}H_{40}O_9NCl$ and Methyl Alcohol—Many repetitions of the study of the action of methyl alcohol on the monochloro derivative have shown that a number of competing reactions occur, with the production of a mixture of substances. The following is a representative experience in this regard and the formulas considered can be only tentative.

0.35 gm. of the monochloro derivative and 10 cc. of dry methyl alcohol were heated at 100° for 18 hours. The light brown solution was concentrated to dryness and the residue was dissolved in chloroform. After removal of free HCl with dilute Na_2CO_3 the chloroform was extracted with 2 per cent H_2SO_4 to remove basic material. The latter was recovered by liberation from the acid solution with an excess of Na_2CO_3 and reextraction with chloroform. This fraction will be treated below. The above chloroform solution containing the neutral material was dried and concentrated, residual chloroform being finally removed with methyl alcohol. The resulting solution, in a volume of about 2 cc., set to a crop of colorless needles. This fraction amounted to 75 mg. and on rapid heating melted with decomposition at 285–290°. The substance contained no Cl. On recrystallization from chloroform-ether the melting point was unchanged and the liberation of acetic acid during the melting could be detected.

$C_{32}H_{39}O_9N$.	Calculated.	C 66.06,	H 6.77,	OCH_3 16.01
	Found.	" 65.55,	" 6.59,	" 15.04

After recrystallization from methyl alcohol it melted with decomposition at 282–284°.

$$[\alpha]_D^{28} = -120° \ (c = 1.14 \text{ in chloroform})$$

Found. C 66.24, H 6.80, OCH_3 14.76

In other experiments the following figures were obtained.

Found.	(*a*)	C 66.34,	H 7.12,	OCH_3 15.82
"	(*b*)	" 65.93,	" 6.71	
"	(*c*)	" 66.20,	" 6.82,	OCH_3 14.40

From the original mother liquor of the above neutral substance successive fractions were obtained which appeared to consist of at least two substances the homogeneity of which was doubtful.

By fractionation from methyl alcohol an intermediate, less soluble fraction was finally obtained as needles which melted at 238–240°.

Found. C 65.22, H 6.87, OCH_3 16.68

In another experiment a corresponding fraction melted at 225–235°.

Found. C 65.32, H 7.12

The most soluble fraction after recrystallization from chloroform-ether melted at 210–211°.

$[\alpha]_D^{28} = -49°$ (c = 0.99 in chloroform)
Found. C 65.56, H 6.92

In a different experiment a corresponding fraction, which melted with decomposition at 205–209° after preliminary sintering, was finally obtained from ether.

This fraction definitely suggests the presence and therefore regeneration of oxodelphinine itself; the analytical figures are given below.

$C_{33}H_{43}O_{10}N$.	Calculated.	C 64.56,	H 7.07,	OCH_3 20.22
	Found.	" 65.03,	" 7.13,	" 19.36

The above high melting neutral substance (m.p. 285–290°) was hydrogenated in acetic acid solution with platinum oxide catalyst under 3 atmospheres pressure. The hydrogenation product crystallized from chloroform-ether in needles which melted slowly at 275–278° after preliminary softening.

$C_{32}H_{45}O_9N$.	Calculated.	C 65.38,	H 7.72
	Found.	" 66.07,	" 7.71
	"	" 65.88,	" 7.86

The chloroform solution containing the basic fraction on concentration to a small volume and careful addition of ether crystallized on rubbing. The yield was 25 mg. In another experiment 0.6 gm. of chloro compound gave 37 mg. of this base. It melted at 218–220° after preliminary sintering.

$C_{32}H_{39}O_9N$.	Calculated.		C 66.06,	H 6.77,	OCH_3 16.01
$C_{31}H_{37}O_9N$.	"		" 65.57,	" 6.57,	" 10.94
	Found.	(a)	" 66.03,	" 7.09,	" 14.40
	"	(b)	" 65.86,	" 6.98,	" 12.62, 12.27

A small sample of this base readily gave a crystalline nitroso derivative which after recrystallization from dilute acetone melted at 236–238° after preliminary sintering.

$C_{32}H_{38}O_{10}N_2$.	Calculated.	C 62.92, H 6.28, OCH_3 15.25
$C_{31}H_{36}O_{10}N_2$.	"	" 62.38, " 6.08, " 10.41
	Found.	" 62.16, " 6.58, " 11.81

In general, the varying analytical figures make fairly certain the doubtful homogeneity of the above substances. Their precise interpretation must await a later opportunity.

The Substance $C_{32}H_{39}O_8NCl_2$ (?)—The effect of longer action of HCl was shown as follows:

1 gm. of α-oxodelphinine was sealed in a tube with 20 cc. of methyl alcohol saturated at 0° with HCl. The solution was allowed to stand at room temperature for 7 days and was then worked up as in the preceding case. The basic fraction was negligible. After concentration of the chloroform solution of the neutral fraction to 5 cc. and careful treatment with ether, crystallization could be started, and a paste gradually formed. The product was collected with a 1:1 chloroform-ether mixture and amounted to 0.21 gm. Succeeding fractions up to a total of 0.4 gm. were obtained by manipulation of the mother liquor.

The first fraction was recrystallized by concentration of its acetone solution and formed needles which melted gradually with effervescence at 225–227° after preliminary sintering.

$C_{32}H_{39}O_8NCl_2$.	Calculated.	C 60.36, H 6.18, Cl 11.15, OCH_3 14.63
$C_{30}H_{37}O_7NCl_2$.	"	" 60.58, " 6.28, " 11.93, " 15.66
	Found.	" 61.00, " 6.69, " 10.88, " 14.92

In an experiment in which the reaction was allowed to stand for 11 days a similar result was obtained.

Found. C 60.90, H 6.03, Cl 10.58, OCH_3 15.26

β-Oxodelphinine and Methyl Alcoholic HCl. Methylbenzoyl-β-Oxodelphonine—A solution of 0.9 gm. of the β derivative in 18 cc. of 4 per cent methyl alcoholic HCl was heated at 100° for 16 hours. The clear solution after concentration was separated as in other cases into basic and neutral fractions. The basic fraction which was relatively small could not be crystallized; a small fraction which crystallized from ether was found to be neutral and iden-

tical with the accompanying neutral substance. It was probably carried along by virtue of its solubility in the solvents used.

The neutral fraction crystallized under ether as delicate needles. This substance proved to be quite soluble and was most readily recrystallized by solution in chloroform and then concentration and finally by addition of ether and concentration to remove all chloroform. It melted not sharply at 182–185°.

$[\alpha]_D^{30} = +27°$ (c = 0.555 in methyl alcohol)

$C_{32}H_{43}O_9N$.	Calculated.	C 65.60, H 7.40
	Found.	" 65.64, " 7.55

In another experiment 0.1 gm. of β-oxodelphinine was allowed to stand for 16.5 hours in a tube with 2 cc. of methyl alcohol saturated at 0° with HCl. The clear solution yielded several fractions amounting to 50 mg. which were halogen-free and possessed all properties of unchanged starting material.

$C_{33}H_{43}O_{10}N$.	Calculated.	C 64.56, H 7.07, OCH_3 20.22
	Found.	" 64.28, " 7.15, " 19.60

In like manner the above pentamethoxyl compound was recovered unchanged after similar treatment with saturated methyl alcoholic HCl. The recovered substance melted at 180–183°.

$C_{32}H_{43}O_9N$.	Calculated.	C 65.60, H 7.40
	Found.	" 65.37, " 7.20

Pyro-α-Oxodelphinine and Hydrochloric Acid—Pyrooxodelphinine was treated at room temperature with 10 parts of HCl (1.19). When worked around with a rod it did not dissolve but gradually became more voluminous. After standing about 18 hours, the mixture was diluted with water. The substance collected proved to be very sparingly soluble in the usual solvents. It formed minute needles and four sided platelets which showed a decomposition point of 318–320° after preliminary darkening.

$C_{30}H_{36}O_7NCl$.	Calculated.	C 64.54, H 6.51, Cl 6.36, OCH_3 16.69
	Found.	" 64.43, " 6.54, " 6.10, " 16.56

0.51 gm. of pyro-α-oxodelphinine in a tube with 10 cc. of methyl alcohol saturated at 0° with HCl was allowed to stand at 20–25° for 21 hours. The clear solution after removal of the reagent was separated as in the previous cases into neutral and

basic fractions, with ample amounts of chloroform as solvent. The basic fraction was again negligible in amount. When the neutral chloroform solution was concentrated to 3 cc. and allowed to stand, a first fraction separated consisting of globular aggregates of crystals; 0.27 gm. was collected. Additional material was obtained from the mother liquor. The first fraction was dissolved in 50 cc. of boiling chloroform; on concentration to about 5 cc. and addition of several volumes of ether prompt separation of aggregates of wedge-shaped crystals took place.

This substance began to discolor above 240°, became progressively darker, and finally sintered to a dark mass above 260–265° with no definite melting point.

$C_{29}H_{33}O_6NCl_2$.	Calculated.	C 61.90,	H 5.92,	Cl 12.61,	OCH_3 11.04
	Found.	" 62.22,	" 6.18,	" 12.41,	" 11.45

In an experiment in which 0.2 gm. of pyro-α-oxodelphinine was allowed to react with the reagent as above but for 7 days at room temperature, again almost all the material remained in the neutral fraction. But in this case only 10 mg. of substance were directly obtained from the chloroform solution. However, careful treatment with ether yielded successive crystalline fractions which when once obtained appeared to be sparingly soluble in chloroform. The combined fractions (0.1 gm.) were recrystallized from chloroform and ether (1:1) and formed minute wedge-shaped or triangular crystals which decomposed at 250–253° after preliminary darkening.

Found. C 61.16, H 6.07, Cl 12.90

40 mg. of the first dichloro derivative were heated in 10 cc. of methyl alcohol for 23 hours. The concentrated mixture was dissolved in chloroform and shaken with dilute Na_2CO_3. The residue from the chloroform solution crystallized readily from methyl alcohol as glistening compact aggregates of prisms which melted at 278–280° after preliminary softening. This substance contained no Cl and appeared to be identical with the substance (1) previously obtained by heating pyro-α-oxodelphinine with methyl alcoholic HCl.

$C_{31}H_{39}O_8N$.	Calculated.		C 67.23,	H 7.10,	OCH_3 22.40
	Found.	(a)	" 67.47,	" 6.87,	" 22.00
	"	(b)	" 67.42,	" 6.77	

Hydrogenation of the Substance $C_{29}H_{33}O_6NCl_2$—50 mg. of the dichloro derivative were hydrogenated with 20 mg. of platinum oxide catalyst in acetic acid solution. Although most absorption occurred during the 1st hour, hydrogenation was allowed to continue for 18 hours. The apparent absorption was from 4 to 5 moles. After removal of the solvent, the residue was dissolved in chloroform and shaken with dilute Na_2CO_3 solution. The product crystallized from ether as small glistening prisms which melted with effervescence at 216–218°. It still gave a strong halogen test.

$C_{29}H_{39}O_6NCl_2$.	Calculated.	C 61.24, H 6.92
	Found.	" 61.55, " 7.20

α-Oxodelphinine and Nitric Acid—α-Oxodelphinine was dissolved in 10 parts of HNO_3 (1.42) at 20–25°. After 18 hours, dilution of the clear solution caused copious crystallization of a practically colorless product. It was recrystallized by solution in chloroform and concentration after addition of alcohol. It was necessary to remove all the chloroform; otherwise the analytical results were affected by tenaciously held solvent. The substance formed both narrow and broad microneedles which melted with decomposition at 271–273°.

$C_{32}H_{41}O_{10}N$.	Calculated.	C 64.07,	H 6.89,	N 2.34,	OCH_3 15.53
	Found.	" 63.98,	" 6.79,	" 2.51,	" 15.80
	"	" 63.92,	" 6.71		

Pyro-α-Oxodelphinine and Nitric Acid—Pyro-α-oxodelphinine was dissolved in 10 parts of HNO_3 (1.42). The clear solution after 15 minutes at 20° gave on dilution a crystalline product. This was recrystallized by solution in chloroform and addition of alcohol with subsequent concentration to remove the chloroform. It formed characteristic compact triangular or trapezoidal prisms which gave a micro melting point of 309–310°.

$C_{30}H_{37}O_8N$.	Calculated.	C 66.75,	H 6.91,	N 2.60,	OCH_3 17.26
	Found.	" 66.45,	" 6.87,	" 2.77	
	"	" 66.36,	" 7.01		
	"	" 66.24,	" 7.16,		OCH_3 17.59

When pyro-α-oxodelphinine was somewhat more vigorously treated with nitric acid, a more extensive change occurred which involved cleavage of an additional OCH_3 group and possibly loss of a CH_2 group.

A solution of 0.1 gm. of substance in 1 cc. of HNO_3 (1.42) was kept at 49–51° for 1 hour. During this time nitric oxide fumes appeared. After chilling and dilution an amorphous precipitate crystallized. On recrystallization from a small volume of acetone it formed minute, colorless wedge-shaped crystals which sintered to a resin above 200° and melted gradually at 235–240°.

For analysis it was dried *in vacuo* at 120°.

$C_{28}H_{33}O_8N$.	Calculated.		C 65.72,	H 6.51,	N 2.74,	OCH_3 12.14
$C_{29}H_{35}O_8N$.	"		" 66.25,	" 6.72,	" 2.67,	" 11.81
	Found.	(*a*)	" 65.93,	" 6.25,		" 11.32
	"		" 65.79,	" 6.25		
	"	(*b*)	" 65.63,	" 6.67,		OCH_3 11.45
	"	(*c*)			N 3.09	

When the substance $C_{30}H_{37}O_8N$ was treated with HCl (1.19), it did not appreciably dissolve but gradually changed its appearance on standing at 25°. After 18 hours the acid was carefully diluted with water. After solution in a large volume of an alcohol-chloroform mixture (1:1) and concentration to remove the chloroform the product crystallized as minute triangular or trapezoidal crystals which sintered under the microscope above 242° and lost transparency above 272° but failed to show a real melting or decomposition point on further heating.

$C_{30}H_{36}O_7NCl$.	Calculated.	Cl 6.36,	$3(OCH_3)$ 16.69
	Found.	" 6.86,	" 16.05

Delphinine and HNO_2—1.05 gm. of delphinine were dissolved in 40 cc. of 25 per cent acetic acid and treated with 20 cc. of 30 per cent sodium nitrite solution. The mixture was heated on the water bath for 2 hours during which time a small amount of sparingly soluble crystals separated. After cooling, the latter were collected with water. The yield was 0.11 gm. This substance which was neutral was recrystallized by solution in acetone and concentration to crystallization. It formed minute, short, mostly four sided crystals which melted with decomposition at 240–241° after preliminary softening.

It gave a gradually developing weak nitroso test with phenol and H_2SO_4.

$C_{33}H_{44}O_{10}N_2$.	Calculated.	C 63.02,	H 7.06,	N 4.46,	OCH_3 19.73
	Found.	" 62.85,	" 7.11,	" 4.19,	" 20.07
	"	" 63.24,	" 6.94		

The (N)CH_3 determination (0.55) showed but a fraction of a mole. Calculated, (N)CH_3 2.39.

0.0105 gm. of substance was refluxed with 0.0958 cc. of 1.01 N NaOH and an equal volume of alcohol for 2 hours and titrated back against phenolphthalein. Found, 0.0388 cc. Calculated for 2 equivalents, 0.0334 cc.

The dilute acetic acid filtrate from the nitroso derivative was carefully neutralized and then made definitely alkaline with dilute NaOH and extracted with chloroform. The washed and dried extract after concentration to a few cc. and addition of a considerable volume of ether gave an appreciable colorless amorphous precipitate which was collected with ether. The ether filtrate on concentration to small bulk yielded minute prisms, which for recrystallization were dissolved in chloroform and the latter driven off with ether. The base formed small triangular prisms and needles. The separation was aided by cautious addition of petroleum ether. The yield was 0.3 gm.

The substance melted with slow effervescence at 180–182°; occasionally samples were obtained which melted somewhat higher; *viz.*, 193–195° after preliminary sintering.

$$[\alpha]_D^{20} = +7° \ (c = 1.1 \text{ in absolute alcohol})$$

$C_{33}H_{45}O_{10}N$

Calculated.		C 64.35,	H 7.37,	N 2.28,	OCH_3 20.17,	(N)CH_3 2.44
Found.	(a)	" 64.50,	" 7.46,	" 2.44,	" 20.10,	" 1.43
"	(b)	" 64.10,	" 7.42			
"	(c)	" 64.52,	" 7.41			

γ-Oxodelphinine—The above base, $C_{33}H_{45}O_{10}N$, was oxidized in acetone solution containing acetic acid with $KMnO_4$ by the usual procedure. From 0.25 gm. successive fractions of neutral oxidation product were obtained which amounted to 0.175 gm. It crystallized from ether as four sided platelets or compact prisms which melted at 226–229°. Superficially it was indistinguishable from β-oxodelphinine and gave no depression of melting point when mixed with the latter.

$$[\alpha]_D^{20} = +32° \ (c = 0.63 \text{ in } 95 \% \text{ alcohol})$$

$$[\alpha]_D^{20} = +40° \ (c = 0.94 \text{ in acetic acid})$$

$C_{33}H_{43}O_{10}N$. Calculated. C 64.56, H 7.07
Found. " 64.55, " 7.01

0.14 gm. of γ-oxodelphinine was heated in 3 cc. of 4.3 per cent methyl alcoholic HCl at 100° for 17 hours. The clear solution

after concentration to dryness *in vacuo* was treated with excess dilute Na_2CO_3 and ether. During the ether extraction a portion of the neutral reaction product crystallized. Extraction of the ether filtrate with dilute acid yielded but a negligible basic fraction. From the neutral ether solution more of the above crystalline neutral substance was recovered.

Recrystallization was effected by dissolving in methyl alcohol, then adding water, and boiling off most of the former. The product separated from the aqueous solution as square ended needles which melted gradually at from 170–177°. On addition of petroleum ether to the concentrated ether solution it slowly separated as woolly needles which sintered above 140° to a resin which melted at 184–188°. When mixed with methylbenzoyl-β-oxodelphonine from β-oxodelphinine it showed no depression of melting point. However, the rotation differed considerably from that of the latter.

$$[\alpha]_D^{30} = +5° \ (c = 0.615 \text{ in methyl alcohol})$$

For analysis the substance was dried at 110° and low pressure.

$C_{32}H_{43}O_9N$.	Calculated.	C 65.60, H 7.40
	Found.	" 65.60, " 7.42

Benzoyldelphinine—1 gm. of the alkaloid dissolved in 20 cc. of dry pyridine was treated with 2 cc. of benzoyl chloride. After 20 hours at 20° the mixture was diluted with water and after addition of excess Na_2CO_3, pyridine was removed *in vacuo*. The diluted residue was extracted with chloroform. The latter gave a residue which crystallized in dilute methyl alcohol. The product was recrystallized from 95 per cent alcohol and formed needles which melted at 171–173°.

For analysis it was dried at 120° and low pressure.

$C_{40}H_{49}O_{10}N$.	Calculated.	C 68.24, H 7.02
	Found.	" 67.77, " 7.05

Benzoyloxodelphinine—0.12 gm. of benzoyldelphinine was oxidized in 12 cc. of acetone containing 0.1 cc. of acetic acid with 80 mg. of $KMnO_4$. Oxidation occurred only gradually at room temperature and after 18 hours an appreciable excess of reagent persisted. The filtrate after concentration was treated with water and chloroform. The chloroform extract was successively washed

with dilute H_2SO_4 and then dilute Na_2CO_3 and dried. The chloroform solution left a resin on evaporation which crystallized in methyl alcohol. It formed delicate needles which melted gradually at 185–187°.

$C_{40}H_{47}O_{11}N$.	Calculated.	C 66.91, H 6.60
	Found.	" 66.96, " 6.69

No attempt was made to determine the production of isomers as in the case of the oxidation of delphinine itself.

SUMMARY

In an attempt to find a means for the controlled degradation of the delphinine molecule a study has been made of the effect of hot methyl alcohol, methyl alcoholic HCl, and nitric acid on delphinine itself and on α- and β-oxodelphinine and pyrooxodelphinine. The reactions have been found to take a different course in each case and are affected by temperature, strength of reagents, and duration of the reaction.

The reactions have to do essentially with replacement of acetoxyl by methoxyl or of one or more methoxyl groups by OH or Cl. In the last case the action of strong methyl alcoholic HCl extends from little or no effect on delphinine to replacement of two methyl groups in pyrooxodelphinine by 2 Cl atoms. In the latter case the halogen could not be replaced by hydrogenation.

Nitrous acid acts on delphinine to give the nitroso derivative of an apparently isomeric secondary base and also a hydroxydelphinine. The latter on oxidation gave a new or γ-oxodelphinine.

A benzoyldelphinine and its oxidation to benzoyloxodelphinine have been described. Their behavior on pyrolysis has been studied in the investigation of the nature of the pyro derivatives.

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THE ACONITE ALKALOIDS

III. THE OXIDATION OF ACONITINE AND DERIVATIVES WITH NITRIC ACID AND CHROMIC ACID

By WALTER A. JACOBS AND LYMAN C. CRAIG

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

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In earlier studies on the chemistry of aconitine and related alkaloids a number of observations have accumulated, especially in the case of the former, which present certain inconsistencies. Although the changes produced by certain reagents do not represent extensive degradation of the alkamine portion of the molecule, it appears that a number of reactions involving different groups have occurred more or less simultaneously, so that mixtures of reaction products were apt to result. This combined with the difficulty or uncertainty in the isolation of individual substances from the mixture and the tendency to separate with solvent has contributed to some confusion. We have previously (1) discussed the case of oxonitine and suggested a revision of the formulas proposed for it. A return to a further discussion of this formulation and that of its companion oxidation product, oxoaconitine, first described by us will be made in a subsequent paper.

In a parallel study of the behavior of aconitine and delphinine and derivatives towards certain reagents we have had occasion to reconsider and repeat some of the earlier observations on record in regard to the former. Thus, the so called nitrosodicarboxylic acid, $C_{22}H_{26}O_{11}N_2$, first obtained by Brady from aconitine with hot HNO_3 (2), has recently been shown by Suginome (3) to be a product also from oxonitine and mesaconitine. The latter author, however, described it as a nitronitrosomonocarboxylic *acid*, $C_{31}H_{33}O_{13}N_3$, which he called nitronitrosoaconitinic acid, and which retained both benzoyl and acetyl groups intact but only three methoxyl groups and no N-alkyl group. This substance yielded on saponification a so called nitronitrosoaconinic acid, $C_{22}H_{27}O_{11}N_3$.

Somewhat before this, Lawson (4) had reported results of a similar study of the oxidation of both aconitine and oxonitine with HNO_3. However, from each he believed that he had obtained different products. From the former a crystalline *acid*, $C_{31}H_{35}O_{13}N_3$, was described containing three methoxyl groups and no N-alkyl which after saponification gave an *acid*, $C_{22}H_{29}O_{11}N_3$. From oxonitine the product was described as an acid with a formula, $C_{31}H_{35}O_{14}N_3$. Finally, with chromic acid Lawson obtained a base, aconitoline, $C_{30}H_{37}O_9N$, which retained the N-alkyl group but only three methoxyl groups and which on saponification was supposed to lose only the benzoyl group to give a base which was isolated as the HCl salt; *viz.*, $C_{23}H_{33}O_8N \cdot HCl \cdot 3H_2O$. With HNO_3 aconitoline was reported in turn to give an acid, $C_{29}H_{33}O_{13}N_3$, also containing only three methoxyl groups but with no N-alkyl group.

In repeating the above work under the conditions employed respectively by Suginome and by Lawson, we have become convinced that both aconitine and oxonitine, as stated by the former, indeed yield the same nitronitroso derivative but that the formula is $C_{31}H_{35}O_{13}N_3$ and not that given by him. This formula was adopted by Lawson in the special case of his product from aconitine. The analytical results published by Suginome on the anhydrous material actually agree better with this formula than with that of $C_{31}H_{33}O_{13}N_3$ adopted by him. Therefore its saponification product should be $C_{22}H_{29}O_{11}N_3$. In addition we have found that aconitoline, contrary to Lawson, also yields the same nitronitroso compound, $C_{31}H_{35}O_{13}N_3$. This at once forces a changed interpretation of the formula of aconitoline itself, for which we now propose $C_{33}H_{41}O_{10}N$. This conforms with the older results of Schulze (5) who had oxidized the alkamine aconine to a base, $C_{24}H_{35}O_8N$. The base obtained by us on saponification of aconitoline was found to be identical with that obtained according to Schulze from aconine. As described by him, it yielded a methiodide, $C_{24}H_{35}O_8N \cdot CH_3I$. Strangely enough, we have found that aconitoline itself does not react readily with methyl iodide, at least under the gentle conditions which so easily gave the above quaternary salt from its saponification product.

By following also the essential procedure used by Schulze for the oxidation of aconine to the above base in aqueous solution, we have obtained aconitoline from aconitine, although in poorer yield

than by Lawson's method in acetone solution. Retention of two free OH groups in aconitoline is indicated by the fact that its saponification product, $C_{24}H_{35}O_8N$, gives a tetraacetyl derivative according to Schulze, although the active H determinations reported by Lawson on aconitoline indicated only one OH group. This is a point which will have to be rechecked when opportunity is presented.

Further, it has been found that oxoaconitine with HNO_3 also yields, although apparently somewhat less readily than oxonitine, the above nitronitroso derivative, $C_{31}H_{35}O_{13}N_3$. In the case of both oxonitine and oxoaconitine it has been possible by more gentle treatment with HNO_3 to intercept the reaction at an intermediate stage in which only a nitro group has been introduced with simultaneous loss of a methoxyl group. Analysis of the substance from oxonitine suggests either a formula $C_{32}H_{36}O_{13}N_2$ or possibly $C_{33}H_{38}O_{13}N_2$. The last, however, would not conform to a formula $C_{33}H_{32}O_{12}N$ for oxonitine itself. An analogous substance, $C_{33}H_{38}O_{13}N_2$, was similarly obtained from oxoaconitine which superficially at least appeared indistinguishable in character from the substance from oxonitine. Both of these substances with hot HNO_3 were converted by nitrosation and simultaneous degradation into the above nitronitroso derivative $C_{31}H_{35}O_{13}N_3$.

Finally, a substance was also described by Lawson as a nitroso compound, $C_{31}H_{40}O_{12}N_2$, resulting from the action of nitrous acid on aconitine itself and which was briefly stated to yield with nitric acid the above nitronitroso acid. However, no analyses of the latter were given. The production of such a nitroso derivative has been confirmed by us as well as its very ready conversion by HNO_3 to the substance $C_{31}H_{35}O_{13}N_3$. But its analysis appears to accord best with a formula $C_{34}H_{44}O_{13}N_2$. Although it does not exhibit an N-alkyl group, it still retains all four methoxyl groups. Its production, therefore, resembles that of the nitroso derivative $C_{33}H_{44}O_{10}N_2$ from delphinine (6) which, however, did not undergo the apparent additional oxidation of a CH_2 group to CO which obviously occurs in the production of the aconitine derivative.

The exact interpretation of interrelationships of the above oxidation products is still difficult and must await the accumulation of additional data. It is apparent, however, that the nitronitroso derivative, $C_{31}H_{35}O_{13}N_3$, represents a stage beyond all of the above

oxidation products and derivatives, since it is obtained from all of them by the more or less vigorous action of HNO_3. It must be a nitroso derivative of a secondary amine.[1] We have found rather vigorous action of HCl necessary to remove the NO group as reported by Lawson for the production of the base, $C_{31}H_{36}O_{12}N_2$. The latter was very readily reconverted into the nitroso derivative with HNO_2. Apparently a complicated series of steps is involved in the conversion of aconitine into this substance which is intercepted at different stages and in different order in the case of each of the intermediate substances.

For instance, the production of aconitoline, $C_{33}H_{41}O_{10}N$, which differs from aconitine by CH_6O appears to result from the oxidation of a secondary OH group to CO. This must be followed by either separate loss as H_2O of an OH group β to it along with a CH_2 group, or all as methyl alcohol due to the labilizing effect of the CO group.

Triacetylaconitine in which all OH groups have been protected does not react with chromic acid under similar conditions. Delphinine which does not contain two of the hydroxyl groups present in aconitine is similarly not affected by this reagent. That the benzoyl and acetyl groups must occur in the general proximity of the N atom is suggested by the fact that aconitoline shows little tendency if any to quaternary salt formation, unlike its saponification product. The unsaturated character of aconitoline was indicated not only by its ready oxidation with $KMnO_4$ but by hydrogenation, although the hydrogenation product could not be crystallized. The latter yielded on saponification an amorphous substance different from the base, $C_{24}H_{35}O_8N$, given by aconitoline itself.

The first action of HNO_3 on oxonitine or oxoaconitine appears to proceed with oxidative removal of H_2 and introduction of an NO_2 group. This occurs by substitution without loss of carbon except that contained in the methoxyl group which is either removed as methyl alcohol or demethylated independently of the loss of H_2O. Thus oxoaconitine $C_{34}H_{45}O_{12}N$ with loss of CH_6O (perhaps at this stage paralleling the formation of aconitoline

[1] Suginome also adopted the view that his nitronitroso compound was a nitrosamine since it lost the NO group on acetylation. However, the analytical data on his acetyl derivative are not convincing.

from aconitine) and nitration would give $C_{33}H_{38}O_{13}N_2$. The production of $C_{31}H_{35}O_{13}N_3$ from this in turn is the result of an obviously complicated oxidative cleavage of a cyclic amide group with simultaneous nitrosation of a liberated secondary basic group. At the same time degradation occurs with loss of C_2H_3O. This nitronitroso derivative, contrary to the statements of previous workers, does not possess a free carboxyl group. Although it very slowly dissolves on standing in dilute aqueous alkali, it is not dissolved by Na_2CO_3 solution and it cannot be extracted from the chloroform solution with the latter. It is either a lactone or a $=CH \cdot NO_2$ derivative which dissolves in alkali as a salt of the aci- form.

Further work is now in progress which it is hoped will help to establish the exact interrelationship of these substances as well as the exact nature of the groups involved in their transformations.

Henry and Sharp (7) in their study of pseudoaconitine have described the production of several oxidation products which are doubtless analogous to those obtained from aconitine. With chromic acid a weakly basic substance was obtained to which the formula $C_{34}H_{45}O_{11}N$ was ascribed and which while retaining the so called N-alkyl group contained one less methoxyl group. Since pseudoaconitine has veratric acid in place of benzoic acid and contains one less OH group than aconitine, the formula of this substance if exactly analagous to aconitoline should be $C_{35}H_{45}O_{11}N$.

With nitric acid two oxidation products were obtained. One of these to which the formula $C_{33}H_{40}O_{16}N_4$ was ascribed, if analogous to the nitronitroso compound from aconitine, $C_{31}H_{35}O_{13}N_3$, should instead have the formula, $C_{33}H_{38}O_{16}N_4$, since it would not only contain one OH group less than the aconitine derivative but nitroveratric acid in place of benzoic acid. If such conclusions are correct, consistent changes would have to be made also in the formulas of the hydrolytic and other products described by Henry and Sharp.

EXPERIMENTAL

Aconitine and Nitric Acid—0.75 gm. of aconitine was oxidized in 5 cc. of HNO_3 (1.42) according to Brady (2) by heating on the steam bath for 1 hour. The precipitate formed on dilution was crystallized by solution in chloroform followed by addition of

alcohol and concentration in order to remove the chloroform. The melting point depended upon the conditions of crystallization. When the substance separated from the hot alcoholic solution, it formed small faintly yellow four sided prisms which colored above 240°, became dark, and melted at 278° with decomposition. When the separation occurred more slowly from the cool solvent, minute wedge-shaped crystals formed which gradually softened to a resin at 185–200° and then crystallized again and melted at 277–279°. For analysis it was dried *in vacuo* at 120°.

$C_{31}H_{35}O_{13}N_3$.	Calculated.	C 56.60, H 5.37, N 6.39, OCH_3 14.16
	Found.	" 56.39, " 5.38, " 6.49, " 14.16

The procedure described by Lawson (4) was repeated in which 1.1 gm. of aconitine were heated for 2 hours at 100° in 10 parts of HNO_3 (1.2). After dilution the collected precipitate was crystallized first from acetone and then as above. The product was indistinguishable in properties from the above substance.

Found. C 56.40, H 5.11

The substances as obtained directly from aconitine gave the impression of being more homogeneous than those obtained from oxonitine or oxoaconitine as given below.

Oxonitine and HNO_3—0.1 gm. of oxonitine was dissolved in 1 cc. of HNO_3 (1.42) at 25° and kept at this temperature for 1 hour. Liberation of nitric oxide fumes indicated an obvious reaction. On dilution, an amorphous precipitate formed which was collected with water. After solution of the dried material in acetone and concentration a trace of unchanged oxonitine separated. The filtrate on careful dilution gave needles which melted with decomposition at 288–289° after preliminary darkening and sintering. On occasion, however, depending upon conditions, it gradually softened to a paste at 175–195°, resolidified, and decomposed at 280–282°. The substance gave no Liebermann nitroso reaction.

$[\alpha]_D^{25} = +14°$ ($c = 0.57$ in ethyl acetate)

$C_{33}H_{38}O_{13}N_2$.	Calculated.	C 59.08, H 5.71, N 4.18, OCH_3 13.88
$C_{32}H_{36}O_{13}N_2$.	"	" 58.51, " 5.53, " 4.26, " 14.18
	Found. (*a*)	" 58.66, " 5.69
	" (*b*)	" 58.43, " 5.39, N 4.12, OCH_3 14.22

When the reaction was allowed to proceed at this temperature for 17 hours, some contamination with the nitroso derivative given

below was apparent, since the substance gave an appreciable though weak Liebermann test.

Found. C 58.28, H 5.51, N 4.64

0.23 gm. of oxonitine was heated in 3 cc. of HNO_3 (1.42) at 100° for 1.5 hours. The amorphous material obtained on dilution was recrystallized first from dilute acetone (yield 70 mg.) and then from methyl alcohol by boiling down the solution in chloroform with the latter solvent. It formed small faintly yellowish pyramidal prisms or wedges which softened gradually from 175–205° and then resolidified and melted with decomposition at 272–274° after preliminary darkening. The melting point depended greatly upon the rate of heating as well as upon the solvents used for recrystallization.

$$[\alpha]_D^{25} = -31° \ (c = 0.92 \text{ in ethyl acetate})$$

This rotation was taken with the substance that had been dried in the desiccator but recalculated on the anhydrous basis.

For analysis it was dried *in vacuo* at 120°.

$C_{31}H_{35}O_{13}N_3$.	Calculated.		C 56.60,	H 5.37,	N 6.39,	OCH_3 14.16
	Found.	(a)	" 56.64,	" 5.69,	" 6.16,	" 14.05
	"	(b)	" 56.72,	" 5.50		
	"	(c)	" 56.56,	" 5.54		

The substance gave a strong Liebermann test. It is not an acid, since it does not dissolve in dilute Na_2CO_3. It does not immediately dissolve in dilute NaOH solution but only slowly on standing owing to an obvious chemical change. It was also not removed from chloroform solution by extraction with Na_2CO_3 solution.

This nitronitroso derivative was also obtained by substituting the above nitro compound, $C_{33}H_{38}O_{13}N_2$, in the reaction with HNO_3 at 80° for 1 hour. It behaved in the same way in the melting point apparatus and showed identical properties.

Found. C 56.92, H 5.58, N 5.81

Oxoaconitine and HNO_3—A solution of 0.1 gm. of oxoaconitine in 1 cc. of HNO_3 at 25° remained practically colorless after 1 hour. After dilution and collection of the colorless precipitate it was dried and recrystallized from dilute acetone. It formed small,

stout, colorless prisms which melted gradually to a resin at 180–190°. In contrast to the substance from oxonitine, however, it showed no tendency to resolidify and melt again at a higher temperature. Repeated efforts to obtain a substance with such a behavior even by seeding with the oxonitine derivative were not successful. It also appeared to be more stable towards nitric acid than the latter, since, when this reagent was allowed to act on oxoaconitine for 48 hours at 25°, a substance with the same properties was readily obtained which still gave a practically negative Liebermann test.

For analysis both samples were dried *in vacuo* at 120°.

$C_{33}H_{39}O_{13}N_3$. Calculated.	C 59.08,	H 5.71,	N 4.18,	OCH_3	13.88
Found. (a)	" 58.82,	" 5.49,	" 4.19,	"	14.10
" (b)	" 58.86,	" 5.52			

$$[\alpha]_D^{25} = +11.5° \ (c = 1.13 \text{ in ethyl acetate})$$

The experience with HNO_3 under more severe conditions was as follows: 0.25 gm. of oxoaconitine was heated in 2.5 cc. of HNO_3 (1.42) at 100° for 2 hours. The development of nitric oxide fumes was only gradual and not nearly as marked as in the case of oxonitine. The substance obtained from dilute acetone by seeding with material from oxonitine softened to a resin at 180–195°, and then partly resolidified and decomposed at 265–267° after preliminary darkening. It appeared indistinguishable from the oxonitine derivative.

$$[\alpha]_D^{25} = -31° \ (c = 0.9 \text{ in ethyl acetate})$$

This rotation was taken with substance dried in the desiccator but was recalculated on the anhydrous basis.

For analysis the substance was dried *in vacuo* at 120°.

$C_{31}H_{35}O_{13}N_3$. Calculated.	C 56.60,	H 5.37,	N 6.39
Found. (a)	" 56.88,	" 5.57,	" 6.07
" (b)	" 57.05,	" 5.30	

The Substance $C_{31}H_{35}O_{13}N_3$ and HCl—0.1 gm. of the nitronitroso derivative (from oxonitine) was suspended in 2 cc. of absolute alcohol and saturated with HCl with chilling at 0°. The substance finally dissolved as the HCl approached the saturation point. The clear solution was allowed to stand at 25° in a sealed tube for 18 hours and was then concentrated *in vacuo*. After

treatment with an excess of Na_2CO_3 solution the base was extracted with chloroform. The residue from the latter crystallized from alcohol as microneedles or prisms which melted with decomposition at 252–253° after softening above 245°. Lawson reported a melting point of 207° for this base which without analysis was called an amino acid. It proved to be a secondary base since it gave at once the nitroso derivative on treatment with acid and $NaNO_2$.

For analysis the substance was dried *in vacuo* at 120°.

$C_{31}H_{36}O_{12}N_2$.	Calculated.	C 59.21, H 5.77, N 4.46
	Found.	" 59.40, " 6.10, " 4.57

When the nitronitroso derivative was heated in 20 parts of 4 per cent absolute methyl alcoholic HCl at 100° for 18 hours, it was recovered almost entirely unchanged.

Found. C 56.56, H 5.57

Aconitine and Nitrous Acid (*4*)—0.6 gm. of aconitine dissolved in 20 cc. of 25 per cent acetic acid was treated with 10 cc. of 30 per cent $NaNO_2$ solution. After 1½ hours on the water bath and cooling, the crystalline deposit was collected with water. The yield was 0.1 gm. When recrystallized by addition of ether to the concentrated chloroform solution, the analytical results showed tenacious retention of small amounts of solvent. It was accordingly recrystallized from acetic acid as described by Lawson and melted with decomposition at 281°. The Liebermann nitroso test was not marked and the substance showed no appreciable N-alkyl group.

$C_{34}H_{44}O_{13}N_2$.	Calculated.	C 59.27,	H 6.44,	OCH_3	18.03
	Found.	" 59.04,	" 6.54,	"	18.68
	"	" 59.18,	" 6.67,	"	18.22

50 mg. of this nitroso derivative were dissolved in 0.5 cc. of HNO_3 (1.42). After 4 hours at 25° the clear solution was diluted. The collected precipitate was recrystallized from dilute acetone. The yield was 41 mg. It gradually softened to a melt over the range 175–197°.

$C_{31}H_{35}O_{13}N_3$.	Calculated.	C 56.60,	H 5.37,	N 6.39,	OCH_3	14.16
	Found.	" 56.50,	" 5.44,	" 6.18,	"	14.13

Aconitoline—This was prepared essentially as described by Lawson (4) by the oxidation of aconitine in acetone solution with CrO_3. 2 gm. yielded 0.86 gm. which melted at 221–222° after slight preliminary sintering.

$C_{33}H_{41}O_{10}N$.	Calculated.	C 64.78, H 6.76
	Found.	" 64.42, " 6.89
	"	" 64.93, " 6.46
	"	" 64.63, " 6.66

The analytical results reported by Lawson were in close agreement with the above formula.

2.5 mg. were refluxed for 2 hours in a mixture of 0.15 cc. of 0.1 N NaOH and 0.15 cc. of alcohol and titrated back with 0.1 N HCl against phenolphthalein. Found, 0.0875 cc. Calculated for 2 equivalents, 0.0818.

9.2 mg. were refluxed for 2 hours in 0.095 cc. of N NaOH and 0.1 cc. of alcohol and titrated back with N HCl. The color change was gradual but the first fading out of indicator occurred at a point corresponding to a consumption of 0.0414 cc. of NaOH. Calculated for 3 equivalents, 0.0453. Further cautious addition of HCl was followed by successive gradual reappearance of indicator color until the consumption of NaOH was reduced to 0.0354 cc. Calculated for 2 equivalents, 0.0302.

The following procedure based essentially upon that employed by Schulze (5) for aconine was less advantageously used.

1.1 gm. of aconitine were dissolved in a mixture of 8 cc. of 10 per cent H_2SO_4 and 42 cc. of H_2O. To this 0.5 gm. of CrO_3 was added which caused a precipitate of the chromate. On heating on the water bath the latter gradually dissolved as oxidation very slowly occurred. Although the reagent appeared to be used up after several hours, heating was continued for 4.5 hours in all. The cooled acid solution was first extracted with ether and then after being rendered alkaline with Na_2CO_3, was repeatedly extracted with chloroform. The washed and dried extract on concentration and removal of the residual chloroform with alcohol yielded 0.22 gm. of crystalline base. After recrystallization it melted at 221–222° and proved to be identical with the substance obtained by the above procedure.

Found, C 64.49, H 6.61

When aconitoline was heated with methyl iodide at 100° for several hours, no evidence of a reaction was obtained and the base was recovered unchanged.

0.1 gm. of aconitoline was oxidized according to Lawson in 1 cc. of HNO_3 (1.2) at 100°. After 1.5 hours the diluted mixture yielded an amorphous solid which was recrystallized from dilute acetone. It separated as yellowish microcrystals which gradually softened from 175–195°, again crystallized, and then decomposed at 272–273° after preliminary softening and darkening.

$$[\alpha]_D^{25} = -28° \ (c = 0.98 \text{ in ethyl acetate})$$

Recalculated on the anhydrous basis,

$C_{31}H_{35}O_{13}N_3$.	Calculated.	C 56.60, H 5.37
	Found.	" 56.41, " 5.40

The Base $C_{24}H_{35}O_8N$—0.22 gm. of aconitoline was dissolved in 2 cc. of absolute alcohol and treated essentially according to the procedure of Lawson with 1.5 cc. of N sodium ethylate. A reaction quickly occurred with crystallization of a sodium derivative, the nature of which was not determined. After dilution with water and acidification with H_2SO_4, benzoic acid, etc., were removed by extraction with chloroform. It was then made alkaline with Na_2CO_3 and repeatedly extracted with chloroform (best in an extractor) to remove the base. This was finally obtained from alcohol-ether as the HCl salt which when desiccator-dried slowly softened above 214° and finally melted with slow effervescence at 219–220°.

For analysis it was dried *in vacuo* at 120°.

$C_{24}H_{35}O_8N \cdot HCl$.	Calculated.	C 57.40,	H 7.23,	OCH_3 18.55,	Cl 7.07
	Found.	" 56.95,	" 7.34,	" 18.31,	" 6.78
	"	" 57.24,	" 7.03		

This showed no depression of melting point when mixed with the HCl salt prepared by oxidation of aconine according to Schulze (5).

The base liberated from the salt readily reacted when heated with methyl iodide at 100°. It formed fine needles, m.p. 222–225°, from methyl alcohol and ether.

$C_{25}H_{38}O_8NI$.	Calculated.	C 49.40,	H 6.31,	OCH_3 15.33,	$(N)CH_3$ 4.95
	Found.	" 49.57,	" 6.27,	" 16.29,	" 4.48

SUMMARY

A study of the oxidation of aconitine and a number of its derivatives with nitric acid and chromic acid has shown that a complicated series of steps is involved which may be intercepted at different stages or in different orders. The product of vigorous action of HNO_3 on aconitine, oxonitine, oxoaconitine, aconitoline, etc., is a neutral N-nitrosonitro derivative containing only three methoxyl groups with the formula $C_{31}H_{35}O_{13}N_3$ which represents a stage beyond all of the others. On acid hydrolysis this yields the corresponding secondary base $C_{31}H_{36}O_{12}N_2$. Gentler action of HNO_3 on oxonitine and oxoaconitine gives intermediate nitro derivatives with loss of one methoxyl group, respectively $C_{32}H_{36}O_{13}N_2$ (or possibly $C_{33}H_{38}O_{13}N_2$) and $C_{33}H_{38}O_{13}N_2$.

The formula of Lawson's aconitoline, obtained from aconitine with chromic acid, has been revised to $C_{33}H_{41}O_{10}N$ and apparently results from oxidation of a secondary OH group to CO with simultaneous loss of methyl alcohol. On saponification it yields the tertiary base, $C_{24}H_{35}O_8N$, obtained by Schulze from aconine which in turn readily gives a methiodide, in contrast to aconitoline itself.

The production and interpretation of other substances from aconitine and related substances are discussed.

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AN IMPROVED METHOD FOR THE RESOLUTION OF SYNTHETIC ALANINE

By EUGENE PACSU AND JAMES W. MULLEN, 2ND*

(*From the Frick Chemical Laboratory, Princeton University, Princeton*)

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In the course of investigations now being carried out in this Laboratory, it was necessary to obtain pure *d*(−)- and *l*(+)-alanine in relatively large quantities. There are two procedures described in the literature for the resolution of synthetic alanine, both of which were tried out and found unsatisfactory. The present paper deals with a simple, rapid method for a complete resolution that gives considerably higher yields than have hitherto been recorded. Also included are data on the stability of the active alanine to racemization and on the effect of concentration on the specific rotation of the benzoylalanine.

The racemic alanine was prepared according to the excellent method of Kendall and McKenzie (1). As to the resolution, the method of Holmes and Adams (2) involving the use of *l*-menthoxyacetyl chloride was first employed. These authors recommended either 60 per cent ethyl alcohol or a 3:1 mixture of high boiling petroleum ether and ethyl acetate as the solvent. We employed these two solvents as well as pure ethyl acetate and 95 per cent ethyl alcohol and in no case could we get a complete resolution of the *l*-menthoxyacetyl-*dl*-alanine. Using this method, we obtained the best results with 60 per cent ethyl alcohol as the solvent but, even so, after three consecutive recrystallizations from 22, 10, and 5 per cent solutions the specific rotation in acetone only dropped from −76° to −59.7° and the melting point changed from 117–118° to 143–145°. Three further recrystallizations from 5, 3, and 1.5 per cent solutions lowered the specific rotation to −59.2° and changed the melting point to 144–147°. The authors

* Research Assistant on Special Funds from the Rockefeller Foundation.

give $-50.6°$ and 147–148° as the constants for *l*-menthoxyacetyl-*d*(−)-alanine. Also in the above six recrystallizations the yield dropped from 66.4 to 2 gm. Consequently the method was considered impractical and the second procedure as described by Fischer (3) involving the use of the alkaloids brucine and strychnine was tried. Fischer benzoylated the *dl*-alanine and first applied brucine for the isolation of the benzoyl-*d*(−)-alanine and then, by the conversion of the benzoylalanine regenerated from the mother liquor into the strychnine salt, obtained the benzoyl derivative of the *l*(+)-alanine. Following Fischer's instructions implicitly, we could not accomplish a resolution. In most cases the concentrations he specified proved to be too high and all of the brucine salt was thrown out of solution, no fractional crystallization taking place. Using less concentrated solutions we were able to separate the active forms but only at the expense of considerably reduced yields.

However, when strychnine was applied *first* to the benzoyl derivative of *dl*-alanine, practically complete resolution took place after only one crystallization. The final product represented the natural alanine. Attempts to prepare a crystalline strychnine salt of benzoyl-*d*(−)-alanine yielded only sirups. On this depends our method for a complete separation with a minimum number of recrystallizations and high yields. Otherwise the details are essentially those given by Fischer. Fischer's specific rotations of $-9.68°$ and $+9.55°$ for the active alanines in hydrochloric acid as compared to our values of $-10.30°$ and $+10.33°$ under similar conditions indicate that the separation of the two forms was more perfect in the method now given.

The isolation of the active alanines from the active benzoyl derivatives involves hydrolysis by boiling 20 per cent hydrochloric acid. In the removal of the hydrogen chloride from the alanine hydrochloride the procedure has again been improved. Fischer used lead monoxide, precipitating the lead remaining in the filtrate as the sulfide. Not only is this method cumbersome but in the two instances that we applied it the specific rotations of the alanines were lower than in the case of the alanines obtained from the same alanine hydrochlorides by the method we shall give below. Although racemization was not definitely proved to have taken place, there is reason to suspect that it did. Bene-

dict (4) has suggested the use of aniline for the removal of the hydrochloric acid. Application of this method is satisfactory except for consistently low yields. We have established experimental conditions favorable to larger yields. If followed closely they give alanine from the hydrochloride in yields of 90 per cent or better and without racemization.

The suspicion that the prolonged boiling of the benzoyl derivative in 20 per cent hydrochloric acid required for complete hydrolysis might affect the optical activity of the molecule was proved erroneous. When active alanine was subjected to the above conditions for 24 hours, it retained its original activity. However, the heating of the alanine hydrochloride solution with lead monoxide and subsequent treatment with hydrogen sulfide may cause partial racemization. In one case the specific rotation of a sample of *d*(−)-alanine dropped from −10.30°, when prepared from its hydrochloride by the aniline method, to −8.55° when set free from the same hydrochloride by the lead oxide method. The purity of the latter product was proved by electrometric determination of the equivalent weight. In order to prove that partial racemization had taken place, an attempt was made to benzoylate this alanine (−8.55°) and resolve the product by means of strychnine. This led to a benzoylalanine that had a rotation of 0° instead of the expected approximate −30°. Another sample of the same alanine was benzoylated and its derivative showed a specific rotation of −10.5°. The purity of this derivative was checked by electrometric titration and the equivalent weight was found within 0.2 per cent of the theoretical. Since the specific rotation of the pure *d*(−)-alanine is −10.30° and that of its benzoyl derivative is −37.0°, it is seen that an alanine (−8.55°) consisting of 8.5 per cent of the *l*(+) and 91.5 per cent of the *d*(−) form has given rise to a benzoyl derivative (0°) that is completely racemized in one case, and to a benzoyl derivative (−10.5°) that represents a mixture of 35.9 per cent of the *l*(+) and 64.1 per cent of the *d*(−) form in the other case. Two more experiments were set up for the determination of the effect of time on the racemization during benzoylation. After ½ hour in the benzoylating medium an alanine sample that had been obtained from a benzoylalanine with a specific rotation of −37.13° yielded a benzoylalanine with −33.9°. In a second experiment, a sample of the

same alanine after 5 hours under similar conditions gave rise to a benzoyl derivative with a specific rotation of only −30.3°. The purity of both samples of benzoylalanine was tested by titration with 0.1 N potassium hydroxide solution in the presence of phenolphthalein and found to be correct to within 0.5 per cent. Thus again we have evidence of racemization during benzoylation. A thorough study of the conditions of the above effect was not made and no explanation of the varying loss of optical activity is forthcoming. However, the actual isolation of a positively rotating benzoyl derivative from the second experiment through the strych-

TABLE I

*Effect of Concentration of Active Benzoylalanine on Specific Rotation**

$[\alpha]_D$	g	$1/\log [\alpha]_D$	Δ' (0.6486 − $1/\log [\alpha]_D$)	$1/g$	Δ (3.700 − $1/g$)	Δ/Δ'
degrees						
−34.8	0.2703	0.6486	0.0000	3.700	0.000	
+35.2	0.3020	0.6466	0.0020	3.311	0.389	0.194
−35.4	0.3165	0.6456	0.0030	3.160	0.540	0.180
+35.7	0.3502	0.6440	0.0046	2.855	0.845	0.184
+36.0	0.3888	0.6426	0.0060	2.572	1.128	0.188
−36.6	0.5165	0.6396	0.0090	1.936	1.764	0.196
+37.1	0.6681	0.6371	0.0115	1.497	2.203	0.191
Average						0.189

* The rotation of a quantity (g) of benzoylalanine is calculated from the equation, $n = (3.700 - 1/g)/0.095$, where n represents the change of rotation in 0.1° units over the value of $[\alpha]_D = 34.8°$.

nine salt definitely proves that racemization did take place. It has long been known (5–9) that the acetyl derivatives of the amino acids racemize in varying degrees during their formation. However, we believe that this is the first observation of racemization during benzoylation of an active amino acid.

Finally we have observed that the specific rotation of the active benzoylalanine is a function of the concentration, the plot of $1/\log [\alpha]$ against $1/g$ being apparently linear. These relations may be seen from Table I.

In all instances samples (g) of pure $d(-)$- or $l(+)$-benzoylalanine were dissolved in the equivalent quantities of 1 N potas-

sium hydroxide solutions diluted to 10 cc. and the rotations in a 1 dm. tube were observed. The maximum values of about ±37.0° were obtained for 6 to 7 per cent solutions. Fischer's values of ±37.0° were recorded for 10 per cent solutions, again indicating a better separation with our method. Concentration apparently has no significant effect on the specific rotation of alanine itself.

EXPERIMENTAL

Preparation of dl-Alanine—The method of Kendall and McKenzie (1) with acetaldehyde, sodium cyanide, and ammonium chloride was employed with good results. However, we applied aniline (4) for the liberation of the alanine from the hydrochloride. This considerably shortens the time of preparation and gives better yields than those obtained by the lead oxide method.

Preparation of Benzoyl-dl-Alanine—Benzoylation with benzoyl chloride in the presence of excess sodium bicarbonate was carried out exactly as described by Fischer (3). The yield depends on the thorough mixing of the benzoyl chloride and the aqueous solution of the alanine. Vigorous mechanical stirring for a period of 2 hours was found to be effective. For large scale preparation it is advisable to use a continuous extracting apparatus for the removal of the main quantity of the benzoic acid.

Preparation of Benzoyl-l(+)-Alanine—To a hot solution of 268 gm. of benzoyl-*dl*-alanine in 6 liters of water there were added 464 gm. of strychnine. The clear solution was allowed to stand at 0° for 1 day. During this time 342 gm. of the strychnine salt of the benzoyl-*l*(+)-alanine crystallized in the form of well developed plates. The mother liquor was saved for the preparation of the *d*(−)-alanine. The salt was recrystallized from 4 liters of hot water; yield, 278 gm. (75.9 per cent). From the filtrate, on concentration, an additional quantity of 32.7 gm. (8.7 per cent) of pure strychnine salt was obtained.

For the isolation of the benzoyl-*l*(+)-alanine, 246 gm. of the strychnine salt were dissolved in 2 liters of boiling water and the solution was treated with 388 cc. (1 mole) of 1.256 N potassium hydroxide solution, with vigorous stirring. The strychnine was then removed by filtration and 345 cc. of 1.396 N hydrochloric acid were added to the ice-cold filtrate. The solution was concentrated *in vacuo* to 250 cc. and then cooled to 0°. Pure benzoyl-*l*(+)-

alanine separated out in gable-shaped crystals; yield, 77.2 gm. (86.6 per cent). The crystals melted at 148° and had $[\alpha]_D^{20} = +37.12°$ (0.6681 gm. of substance dissolved in 10 cc. of equivalent potassium hydroxide solution; 1 dm. tube; rotation, 2.48° to the right).

Preparation of Benzoyl-d(−)-Alanine—The mother liquor of the crystalline strychnine salt of the benzoyl-*l*(+)-alanine was concentrated *in vacuo* from 6 liters to 1.7 liters, at which point it began to become sirupy. This solution should contain approximately 380 gm. of the strychnine salt of the impure benzoyl-*d*(−)-alanine, corresponding to about 140 gm. of impure benzoyl-*d*(−)-alanine. The latter would require 338 gm. of brucine tetrahydrate (1:1 mole). Therefore, the strychnine was split off by addition of 800 cc. of 1 N potassium hydroxide solution to the 1.7 liter solution and removed by filtration. The filtrate was treated with 800 cc. of 1 N hydrochloric acid, and then 345 gm. of brucine tetrahydrate were added to the hot solution, which, on cooling at 0°, deposited the crystalline brucine salt of the benzoyl-*d*(−)-alanine; yield, 359 gm. (88.2 per cent). The substance is quite pure; recrystallization is unnecessary.

In order to obtain the free acid the brucine salt (292 gm.) was dissolved in 880 cc. of hot water, the brucine split off with 400 cc. of 1.25 N potassium hydroxide solution, filtered at 0°, and the solution treated with 366 cc. of 1.396 N hydrochloric acid. Crystals of the benzoyl-*d*(−)-alanine began to separate after 10 minutes. These were filtered off, the filtrate was concentrated *in vacuo* to 200 cc., cooled, and the crystalline product separated by filtration; total yield, 77.5 gm. (81 per cent) of pure benzoyl-*d*(−)-alanine with a melting point of 148° and $[\alpha]_D^{20} = -36.9°$ (0.7533 gm. of substance dissolved in 10 cc. of an equivalent quantity of potassium hydroxide solution; 1 dm. tube; rotation, 2.78° to the left).

Preparation of l(+)- and d(−)-Alanine—The hydrolysis of the respective benzoyl derivative was carried out by heating the substance with 5 volumes of 20 per cent hydrochloric acid in an open flask that was equipped with a suitable cooler suspended in the air space of the flask. On cooling of the solution the benzoic acid separated out in a crust which was removed by several extractions with ether. The aqueous acid layer was then evaporated *in vacuo*

to a solid. Experiments carried out under various conditions showed that the precipitation of alanine from the hydrochloride in alcoholic solution by means of aniline was practically quantitative provided that no free hydrochloric acid or water was present. It was, therefore, necessary to dissolve the solid residue repeatedly in ethyl alcohol, the solution being evaporated to dryness each time. Finally the alanine hydrochloride was dissolved in 6 times its weight of 95 per cent ethyl alcohol, and then about 2 moles of freshly distilled aniline were added to the solution. The precipitation of the alanine was completed at 0°. The product was washed with ethyl alcohol and finally with ether; yield, over 90 per cent. The $l(+)$-alanine showed $[\alpha]_D^{20} = +10.33°$ (0.4329 gm. of substance dissolved in 10 cc. of 1 N hydrochloric acid; 1 dm. tube; rotation, 0.63° to the right), and the $d(-)$-alanine had $[\alpha]_D^{20} = -10.30°$ (0.4413 gm. of substance dissolved in 10 cc. of 1 N hydrochloric acid; 2 dm. tube; rotation, 1.28° to the left).

TABLE II

Rotation of 2 Gm. of l(+)-Alanine in 50 Cc. of Boiling 20 Per Cent Hydrochloric Acid; 2 Dm. Tube

Time	α_D	$[\alpha]_D^{20}$
hrs.	*degrees*	*degrees*
0	+0.90	+8.55
1	+0.90	+8.55
3	+0.90	+8.55
7	+0.88	+8.35
24	+0.90	+8.55

In order to test the effect of boiling 20 per cent hydrochloric acid on the optical activity of alanine, 2 gm. of slightly racemized $l(+)$-alanine were refluxed in 50 cc. of the acid. Samples were taken out at certain intervals of time and rotations observed in a 2 dm. tube. The samples were returned to the flask and the boiling continued. The results are given in Table II.

It is obvious that no racemization occurred under these conditions.

SUMMARY

An improved method of resolution of synthetic alanine into the active components is given. The yields are much higher than

any previously reported and the time and labor of preparation are also considerably cut down.

Racemization studies show that active alanine is perfectly stable to boiling 20 per cent hydrochloric acid, whereas benzoylation does attack the asymmetric center.

The specific rotation of the benzoylalanine is a function of the concentration.

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CATALASE. III

By JAMES B. SUMNER, ALEXANDER L. DOUNCE, AND VERNON L. FRAMPTON

(*From the Departments of Zoology and Plant Pathology, Cornell University, Ithaca*)

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We reported our first crystalline beef liver catalase to have a *Kat. f.* of 42,000 (1). Later, we obtained values of about 28,000 (2), and still later, values from 30,000 to 35,000 (3). Theorell (4) reports, "It is now definitely established that the liver catalase from cattle, crystallized according to Sumner *et al.*, gives an activity value of *Kat. f.* = 30,000, while the pure catalase from horse liver possesses an activity which is twice as large." Von Euler and Josephson (5) reported one value of 42,000 for non-crystalline horse liver catalase in 1927, and Agner (6, 7)[1] has reported values ranging from 55,000 to 63,000 for his best horse liver catalase preparations, which were non-crystalline. Dounce and Frampton (8) found their first horse liver catalase preparation to have a *Kat. f.* of 50,000 to 55,000. Later on, crystalline horse liver catalase was found to have *Kat. f.* values from 30,000 to 40,000.

In view of these variations in "capability" we have undertaken to prepare a number of samples of crystalline beef liver catalase by the method of Sumner and Dounce, using dialysis for crystallization (3), of crystalline horse liver catalase by an improved method, and of non-crystalline horse liver catalase by Agner's method (6). Two of the Agner preparations have been subsequently crystallized, both before and after adsorption on tricalcium phosphate in the Tswett column, and we have been inter-

[1] Owing to a misunderstanding of his dry weight determination, we have done Agner an injustice in claiming that his figures showed a *Kat. f.* of 38,000 instead of 28,000 for a joint determination with one of us (3, 6) of the *Kat. f.* of crystalline beef liver catalase. The latter figure should stand.

ested in the observation that the increase in *Kat. f.* brought about by crystallizing the material previous to adsorption is practically the same as that brought about by adsorbing the material in the Tswett column. Crystallization of the adsorbed material does not bring about a measurable change in *Kat. f.*

We have found a wide variation of *Kat. f.* values of the various preparations of horse liver catalase, whereas the crystalline beef liver catalase has usually ranged in *Kat. f.* values from 30,000 to 35,000. We have been unable to obtain horse liver catalase by the Agner method of *Kat. f.* value higher than 32,000. One sample prepared by the Agner method, from frozen liver, gave *Kat. f.* of only 25,600. We have found that for a given sample of horse liver, the *Kat. f.* of the catalase prepared from it is the same within the limit of error of the determination, whether the catalase is prepared by the Agner method or by our improved method.

Using our improved method, which will be described, we have been unable to obtain horse liver catalase of *Kat. f.* 50,000 to 55,000, as first reported by Dounce and Frampton (8), although several times we have obtained values of about 40,000.

In order to determine the homogeneity of the various catalase preparations, we have run diffusion experiments, using the Lamm diffusion cell (9), and have normalized the diffusion curves so obtained. We have found that two samples of crystallized horse liver catalase prepared by our improved method, of *Kat. f.* nearly 40,000, were completely homogeneous according to the diffusion method, and that another sample of horse liver catalase, prepared in the same way, with *Kat. f.* of about 30,000, was also homogeneous. A sample of crystalline beef liver catalase of *Kat. f.* 35,000 was found to be nearly homogeneous by diffusion. Thus it appears improbable that the variation in *Kat. f.* is caused by gross contamination of the catalase with some other protein. Figs. 1 and 2 show the ideal diffusion curve and the points of the normalized curves obtained from the line displacement curves for one sample of horse catalase and a sample of beef catalase.

The diffusion constants for horse and beef liver catalase, corrected to 20°, are given in Table I. Sumner and Gralén (10) reported a diffusion constant at 20° for crystalline beef liver catalase of 4.1×10^{-7}, and a molecular weight by the ultracentrifuge of 248,000. Agner (6) reported a diffusion constant

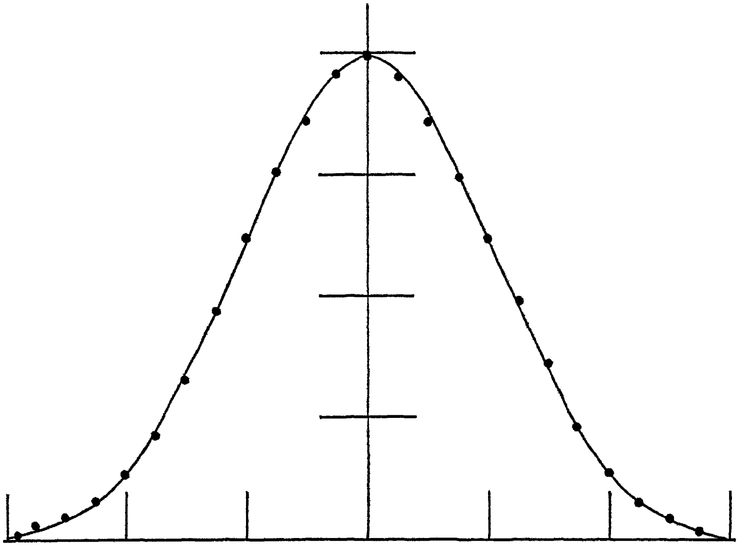

Fig. 1. Normalized diffusion curve for horse catalase. The curve is ideal; the points are experimental values.

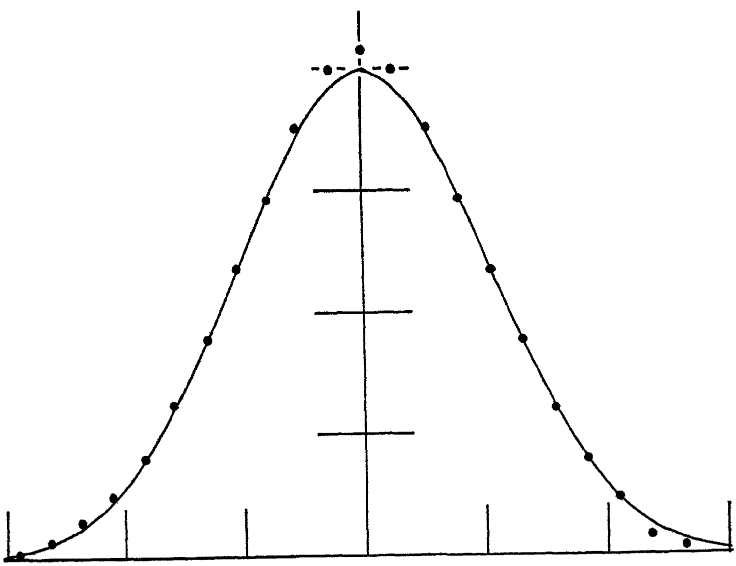

Fig. 2. Normalized diffusion curve for beef catalase. The curve is ideal; the points are experimental values.

for horse catalase of 4.3×10^{-7} and an ultracentrifuge molecular weight of 225,000. Our new diffusion constants for both horse and beef liver catalase are approximately 4.5×10^{-7}. This would change the ultracentrifuge molecular weight of beef catalase from 248,000 to 225,000.[2] The molecular weights, calculated from the Stokes-Einstein formula with the new diffusion constants alone, are given in Table I. The wide divergence between these values and the ultracentrifuge molecular weight is of interest.

TABLE I

Diffusion Constants for Horse and Beef Liver Catalase

	Sample 1. Horse catalase, Dounce method, twice crystallized, fresh liver		Sample 2. Horse catalase, Dounce method, four times crystallized, fresh liver		Sample 10. Beef catalase, Sumner-Dounce method, four times crystallized, fresh liver	
Time ($\times 10^4$), *sec.*	4.89	6.95	5.04	6.34	5.88	6.79
D_{25° ($\times 10^{-7}$)*	5.23	5.12	5.13	5.18		
D_{23° ($\times 10^{-7}$)*					4.76	4.76
D_{20° ($\times 10^{-7}$)†	4.58	4.48	4.49	4.53	4.46	4.46
Radius ($\times 10^{-7}$)†	4.65	4.74	4.74	4.73	4.77	4.77
Particle weight‡	348,000	368,000	368,000	366,000	375,000	375,000

* Corrected for viscosity of solvent.

† Calculated on the assumption that $D = kT/6\pi\eta r$, where k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the solvent, and r is the radius of the particle.

‡ Calculated assuming spherical particles, by the equation $M = (4/3)\pi r^3 \rho N$, where M is the particle weight, r is the radius of the particle, ρ is the density of the protein (calculated from the value of Sumner and Gralén for the partial specific volume of beef catalase), and N is Avogadro's number.

In addition to determining the *Kat. f.* of all our preparations, and the diffusion curves for four of them, we have analyzed twelve samples for hemin iron, iron accompanying the blue material that is split off with the hemin by means of acetone and HCl (3),

[2] Obtained by the equation $M = kTS_{20^\circ}/(D_{20}(1 - \rho_1 v))$, where M is the particle weight, k is the Boltzmann constant, T the absolute temperature, S_{20° the sedimentation constant at 20°, D_{20° the diffusion constant at 20°, ρ_1 the density of water at 20°, and v the partial specific volume of the protein.

TABLE II

Analysis of Horse and Beef Liver Catalases

Sample No.	1	2	3	4	5	6	7	8	8	8	9	10
Catalase	Horse	Horse	Horse	Horse	Horse	Horse	Horse	Horse	Horse	Horse	Horse	Beef
Method	Dounce	Dounce	Dounce	Dounce	Dounce	Dounce	Dounce	Agner, complete	Agner, but not adsorbed	Agner, complete	Agner, complete	Sumner-Dounce
Times crystallized	2	4	4	2			2		2	2	2	
Liver	Fresh	Fresh	Frozen	Fresh	Fresh	Fresh	Fresh	Frozen	Frozen	Frozen	Frozen	Fresh
Kat. f.	37,000	39,000	22,500	36,500	40,000	30,000	55,000	25,600*	26,800	25,600	31,600†	35,500
Hemin iron	0.057	0.052	0.025	0.038	0.06	0.053	0.087	0.036	0.033	0.028	0.044	0.043
Iron with blue material	0.030	0.030	0.067	0.031	0.04	0.048	0.007	0.051	0.053	0.051	0.045	0.037
" in protein	0.004	0.002	0.051	0.007	0.024	0 011	0.058	0.009	0.009	0.006		0.012
Total iron, direct determination	0.106	0.089	0.07	0.091	0.21‡	0.10	0.20	0.073	0.087	0.082	0.103	0.094
Total iron by addition	0.091	0.084	0.143	0.076	0.124	0.112	0.152	0.096	0.095	0.085	0.089	0.092

* *Kat. f.* of this sample before adsorption 21,300.

† *Kat. f.* of this sample before adsorption 28,600; after adsorption and before crystallization 32,600.

‡ The iron content is twice the true value. This indicates the presence of 0.5 per cent ferritin.

total iron, and hemin by rough colorimetry. Table II shows the results of these analyses except for the hemin, which is not included. Hemin determined by colorimetry agrees approximately with hemin calculated from hemin iron.

The total iron of catalase samples from which all of the protein ferritin (11) has been removed is about 0.09 per cent. Some of our values for total iron of horse catalase are too high, owing to incomplete removal of ferritin. Since the latter protein contains about 20 per cent of iron, an amount not exceeding 0.5 per cent would nearly double the value of 0.09 per cent for iron in catalase. Theorell (4) states, "Cow-liver catalase can evidently not be freed from iron impurities by crystallization, and its composition is not completely clear." We know of no evidence to support this statement, and have not obtained high iron values for carefully crystallized beef liver catalase, or indications of the presence of ferritin.

Some of our iron values are too low. We have found that this is caused by incomplete solution of the iron following dry ashing of the sample. We have obtained no low values using a combined wet and dry ashing with sulfuric and nitric acids.

Agner (7) reports that his best horse catalase samples with *Kat. f.* values of 60,000 to 63,000 when analyzed according to our procedure contain 83 per cent of hematin iron, 7 per cent of iron remaining in the protein, and 10 per cent of "blue iron," or iron left in the mother liquor. We have not yet made precise colorimetric determinations of the blue substance, but we have noticed that there is more blue produced by the action of acetone-HCl on catalase of low activity than on catalase of high activity, judging by eye.

From all these facts, we conclude that catalase may exist as several different compounds, similar with respect to protein in any one species, but different with respect to the number of hematin residues and precursors of the blue substance per protein molecule, as postulated in Table III.[3] Our results can be equally well interpreted by assuming the presence of all these catalase

[3] Lemberg suggested this possibility to us in a private communication. We had been considering it for some time before this. We wrote about it to Stern and he has discussed the hypothesis (12) without referring it either to Lemberg or to us.

types, or simply by assuming the presence of completely active Type A and completely inactive Type E in varying proportions. Type E would be analogous to pseudohemoglobin (13, 14).

TABLE III
Hypothetical Catalases

Type of catalase	Theoretical *Kat. f.*	Hematin residues	Precursors of blue substance
A	60,000	4	0
B	45,000	3	1
C	30,000	2	2
D	15,000	1	3
E	0	0	4

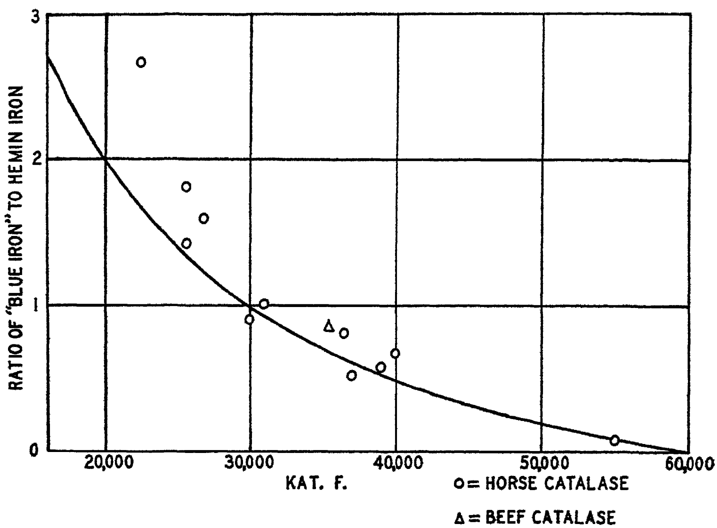

FIG. 3. Ratio of "blue iron" to hemin iron, plotted against *Kat. f.* The points are experimental; the curve theoretical.

We have calculated the ratios of "blue iron" to hemin iron for our various preparations, and have plotted these values against the *Kat. f.* of the sample. This is shown in Fig. 3 in which the curve indicates the theoretical curve that one would obtain by assuming the *Kat. f.* of pure Catalase A to be 60,000 and that of

pure Catalase E to be 0. The points indicate our results. The agreement is fair, in view of the fact that our analyses are of a semiquantitative nature. If this hypothesis is correct, the hematin is the only functional prosthetic group.

The precursor of the blue substance presumably is a biliverdin-iron complex, since Lemberg (15) has recently isolated biliverdin from crystalline beef liver catalase. It is assumed to be produced through the opening up of a hematin ring with the loss of one —CH= group. This might result from enzyme action in the liver of the animal before or after death, since a liver enzyme has been reported (16) which is capable of converting hematin to biliverdin, as well as a copper-activated enzyme of erythrocytes (17) which catalyzes the formation of pseudohemoglobin. On the other hand, it might occur in the process of preparation of catalase. It is of interest to note that frozen liver yielded catalase of the lowest "capability" that we have recorded, but, on the other hand, one particularly fresh sample yielded catalase of *Kat. f.* 32,000 and not 60,000 as might have been expected. A liver from a colt gave catalase of about the same activity as that from an older animal.

Our work, as well as the work of Lemberg, shows that ionic iron accompanies the blue substance. We do not believe that this iron belongs to a group of "iron impurities," *i.e.* ferritin, as Theorell (4) and Agner (7) evidently think.

EXPERIMENTAL

Preparation of Crystalline Horse Liver Catalase—At least 3 kilos of horse liver are ground about ten times in an electric meat grinder. 300 gm. portions are extracted with 400 cc. portions of 40 per cent dioxane. The suspension is transferred to a pail and is allowed to stand at room temperature for about 6 hours. It is then filtered at room temperature through 34 cm. Schleicher and Schüll No. 595 fluted filters, and is next thoroughly chilled in the ice box. Since liver contains about 71 per cent of water, the extract at this point is about 26 per cent dioxane.

After the extract has cooled in the ice box for 2 to 3 hours, 23.3 cc. of dioxane are added for every 100 cc. of extract, with constant stirring. This brings the concentration of dioxane, which has been diluted by the water in the liver, back to about 40 per cent.

The material is next allowed to stand in the ice chest from 6 to 12 hours. If it is allowed to warm to room temperature, much of the catalase may be lost by denaturation. The precipitate which has formed is next filtered off in the ice box, through fluted filters, and refiltered until the filtrate is practically clear. The precipitate is discarded.

Now one adds with stirring 11.1 cc. of dioxane for every 100 cc. of filtrate, and the material is allowed to stand in the ice chest for 3 hours, not longer. The concentration of dioxane is now about 46 per cent, and a large part of the catalase has been precipitated. The precipitate is filtered off through fluted filters with refiltering until a clear filtrate is obtained. This filtration also must be carried out in the ice box. When the material is nearly all filtered, the liquid remaining in the filters is poured upon a new filter paper in order to hasten the filtration.

When the precipitate on the filter papers is well drained from mother liquor, the filter papers are taken out one at a time, spread out on blotter paper or other porous paper, and the precipitate is quickly scraped off with a spatula and is placed in enough water to form a thin cream. This operation must be done quickly. It is worth while to open the filter papers upon a cold surface.

The thin cream which has been formed is now filtered and refiltered until a clear filtrate is obtained; this contains catalase and impurities, among which are ferritin, arginase, and a little hemoglobin. A large amount of glycogen may be present, giving the solution a muddy appearance. This may be destroyed by the addition of a little saliva.

The solution is now dialyzed in the ice chest for 36 hours against several changes of distilled water. The precipitate which forms is filtered off, and one adds to every 10 cc. of filtrate 6.0 cc. of saturated ammonium sulfate. This precipitates the ferritin. After standing for an hour or two in the ice box, the material is filtered, and to every 10 cc. of filtrate one adds 6.0 cc. more of saturated ammonium sulfate. This precipitates the catalase. The precipitated catalase is now centrifuged down and the supernatant liquid is discarded. The precipitate is suspended in just enough water so that it can be transferred to a small dialysis sac. The paste is then dialyzed against many changes of distilled water for 36 hours. During this time, salts diffuse out and the

catalase dissolves, to give a very dark brown or black solution, which later on may deposit a small amount of impurity. After the dialysis, any precipitate which has formed is centrifuged off and the supernatant solution is adjusted to about pH 6.0 by the addition of a very small amount of strong (10 per cent) phosphate buffer. It is essential to keep the concentration of catalase high by avoiding much dilution. Next, one adds gradually and with stirring 2.0 gm. of solid ammonium sulfate to every 10 cc. of solution, and finally enough dioxane to make the solution 3 per cent with respect to dioxane. Now the material is cooled in the ice chest, and saturated ammonium sulfate is added, a few drops at a time, every half hour until crystallization occurs, which may be detected by the silkiness produced on stirring the solution. If any amorphous precipitate forms before the appearance of silkiness, it should be centrifuged off, since it is ferritin and will be hard to remove later.

It is best to take about 2 days for the first crystallization, since otherwise the needles that form are so tiny as to be practically invisible under the high power microscope. In any case, the very small needles that form during the first crystallization are hard to centrifuge down and may require from 2 to 3 hours, with cooling at 20 minute intervals.

To recrystallize, the supernatant is drawn off from the centrifuged crystals, and just enough water is added to form a paste with the crystals that can be transferred to a dialysis sac. The material is then dialyzed for 36 hours, and any precipitate that remains is centrifuged down and discarded. From now on, the procedure is exactly the same as described for the first crystallization.

If just the right amount of ammonium sulfate has been added, and the solution is allowed to stand overnight, rather large crystals can be obtained from the first or second recrystallization. These crystals are either extremely thin long needles, or thin long plates. Some are shown in Fig. 4.

It is of interest that the "thryxotropy" or silkiness produced by stirring the crystals is a much more sensitive test than microscopic observation with direct illumination for deciding whether crystallization has commenced.

As far as we know, it is not possible to crystallize horse liver

catalase without the addition of enough dioxane to give a 3 per cent solution. Less dioxane will not work well, while more results in denaturation of the catalase. Why this should be so, we cannot say. Even several times recrystallized horse liver catalase seems to require dioxane for recrystallization. Horse catalase, unlike beef catalase, cannot be recrystallized by dialysis, since it is too soluble to precipitate.

Preparation of Horse Liver Catalase by Agner's Method—We first employed the method exactly as described by Agner (6). Later we found that his dilutions before precipitating the catalase with ammonium sulfate and with alcohol were excessive, and we therefore used only one-half these dilutions. Our yield was

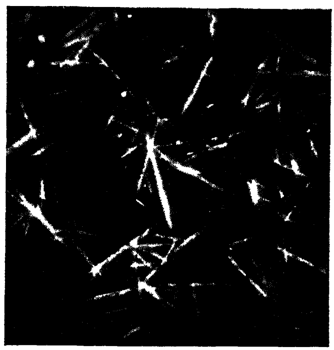

FIG. 4. Crystals of horse liver catalase; × 300

nearly the same as Agner's. About the same amount of once crystallized horse liver catalase is obtained by our method.

Agner does not state how he filters the original water extract of horse liver, or how long this requires. We have generally found it necessary to filter for nearly 2 days in order to obtain sufficient extract. It is possible that during this process, enzyme action disrupts some of the catalase hematin with the production of a considerable amount of the precursor of the blue substance.

Horse liver catalase is a difficult enzyme to prepare in pure condition, in contrast to beef liver catalase which can be prepared by anyone who makes use of the method of dialysis for crystallization already described by us (3). The method of Agner is somewhat cumbersome and time-consuming, while our method is

difficult to describe in precise manner, since slight changes are sometimes necessary with individual samples. Neither of these methods appears to give yields as good as the yield of crystalline beef liver catalase. It might be best for anyone wishing to prepare crystalline horse liver catalase to start out by using Agner's method up to the point of adsorption in the Tswett column, and then carefully to crystallize the material, using our method. This procedure has already been employed by Lemberg (15). The difficulty in our procedure is that the material must not be allowed to stand more than the specified time after addition of the dioxane, and must not be allowed to warm up to room temperature or denaturation will result.

Determination of Total Iron, of Hemin Iron, and of Iron Accompanying the Blue Substance—We have run 5.00 cc. of thoroughly dialyzed horse liver catalase solution (or suspension, in the case of beef liver catalase) into 50 cc. of purified acetone containing 1.0 cc. of concentrated HCl. The acetone must be pure enough so that no color is produced upon addition of HCl to it. From this point on, the separation of the hemin by vacuum distillation of the acetone, after the protein residue is carefully washed with acetone, has already been described (3). Total iron and iron in the hemin and blue substance have been determined by the method of Stugart (18), in which a combined wet and dry ashing with sulfuric and nitric acids is used.

We have found that all the "blue iron" is ionic, in the ferric state. This may be demonstrated by extracting the blue material with isoamyl alcohol, which removes it quantitatively from the water layer. This procedure was discovered by Dr. Olive Hoffman, working in this laboratory. The iron in the aqueous layer is then determined directly, by the method of Stugart (18).

Determination of Diffusion Constants of Horse and Beef Liver Catalase, and of Homogeneity by Diffusion—The measurements of the diffusion constants were made by using the refractive method of Lamm (9). A stainless steel diffusion cell similar to the one described by him was used for these studies. The values of the diffusion constants given in Table I were obtained by following the "analytical" method of calculations.

Determination of the Kat. f. Values—The method of von Euler and Josephson (5) was employed. *K* values were determined

at 3, 6, 9, and 12 minutes, and K_0 was determined by extrapolation. The catalase must not be diluted until just before the analysis is made. For analysis, thoroughly dialyzed samples were used. Since dialysis causes beef catalase to crystallize, a suspension is made and the required amount of this is pipetted into about 5 cc. of water, to which is then added enough NaCl to make the solution 10 per cent, and also a few drops of pH 7.4 phosphate buffer. The dissolved catalase is then diluted as required. An aliquot of the original suspension is used for the dry weight determination. Since horse liver catalase remains soluble after prolonged dialysis, this preliminary solution of crystals before dilution is not required. For most reproducible results, K_0 should lie between 0.025 and 0.04.

SUMMARY

1. The *Kat. f.* or "capability" of crystalline beef liver catalase has been found to vary from 28,000 to 35,000, while that of crystalline horse liver catalase has been found to vary from 22,000 to 55,000.

2. The *Kat. f.* of various catalase preparations has been found to follow inversely the ratio of "blue iron" to hemin iron. This leads us to consider as possible the hypothesis that catalase activity depends upon the number of hematin groups in the molecule, and that the blue substance represents changed hematin prosthetic groups which result in lowering the activity. This hypothesis has been independently suggested by Lemberg in a private communication.

3. The total iron of horse and beef liver catalase is about 0.09 per cent. The total iron is equal to the sum of the hemin iron and the "blue iron."

4. Three samples of crystalline horse liver catalase of differing *Kat. f.* values have been shown to be homogeneous by diffusion. One sample of crystalline beef liver catalase has been shown to be nearly homogeneous by diffusion. The diffusion constant of both beef and horse liver catalase is 4.5×10^{-7} at 20°.

5. Preparation of horse liver catalase by the Agner method, starting with a given sample of liver, has been found to yield catalase of the same *Kat. f.* as that obtained by our method. The Agner catalase can be crystallized before or after adsorption on

tricalcium phosphate in a Tswett column. The increase in *Kat. f.* produced by adsorption is practically the same as that produced by crystallizing the unadsorbed material.

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A RAT ASSAY METHOD FOR THE DETERMINATION OF RIBOFLAVIN*

By J. R. WAGNER,† A. E. AXELROD,‡ M. A. LIPTON, and C. A. ELVEHJEM

(*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*)

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The recognition of riboflavin as an essential dietary constituent has stimulated many attempts to develop a reliable animal assay method for this vitamin. Such an assay requires the development of a ration deficient in riboflavin but adequate in all other factors required by the experimental animal. Bourquin and Sherman (1), Munsell (2), György (3), Day and Langston (4), Supplee *et al.* (5), Hamilton and Mitchell (6), Randoin *et al.* (7), Lindholm (8), and Carlsson and Sherman (9) have proposed rations for the assay of riboflavin with rats. The earlier rations gave limited growth when supplemented with adequate riboflavin, suggesting a deficiency of other dietary factors. The rations proposed by the later workers have supported growth approaching 3 gm. per day. El-Sadr, Macrae, and Work (10) have proposed a ration which is said to maintain growth of 30 to 35 gm. per week when supplemented with riboflavin. This ration has thus far been employed only in the assay of milk.

Early studies in this laboratory (Elvehjem, Koehn, and Oleson (11)) resulted in the production of a ration (No. K_{12}) for the study of the vitamins of the B complex. Frost and Elvehjem (12) showed this ration to be deficient in both riboflavin and factor W.

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† Wilson and Company Fellow.

‡ Commercial Solvents Fellow.

A later modification of this ration resulted in the reduction of the white corn to a level of 6 per cent, and it was shown that under these conditions the ration was deficient in vitamin B_6 and pantothenic acid (Black, Frost, and Elvehjem (13)) in addition to factor W and riboflavin. With the observations of these workers in mind we have prepared a ration which is extremely low in riboflavin and which supports normal growth when adequate riboflavin is added. This ration has been successfully applied to the determination of riboflavin in a wide variety of natural products (Table I).

The butter fat for this ration is prepared by washing melted butter four times with hot water in a separatory funnel. Some

TABLE I

Riboflavin-Free Basal Ration K_{21}

Dextrin	65 parts
Casein (Labco)	18 "
Salts I	4 "
White corn	6 "
Butter fat	3 "
Cod liver oil	2 "
Corn oil	2 "
Thiamine*	200 γ per 100 gm.
Vitamin B_6*	200 " " 100 "
Nicotinic acid	2500 " " 100 "
Riboflavin-free liver extract	≈4% of starting material

* We are indebted to Merck and Company, Inc., for generous supplies of vitamin B_1 and vitamin B_6.

variation in the fat content of the ration is permissible. Excellent growth is obtained when 5 per cent butter fat is used and the corn oil is omitted. However, after several months upon this ration some evidence of a Burr deficiency (14) is obtained. Similarly excellent growth is obtained when the butter fat is omitted and either 3 or 5 per cent corn oil is used. Salts I have been described by Phillips and Hart (15).

The basal ration containing the proper amounts of dextrin, salts, white corn, and casein plus the thiamine, nicotinic acid, and vitamin B_6 may be prepared in bulk and may be stored safely. The liver concentrate, cod liver oil, and fat should be added to each week's supply.

Preparation of Riboflavin-Free Liver Extract—Suitable starting materials for this extract are liver powder (No. 1-20) or liver Fraction B (The Wilson Laboratories). Other liver preparations have yielded less suitable concentrates. The latter product is a desiccated water-soluble powder obtained as a by-product in the concentration process for pernicious anemia preparations. The former product represents a 20-fold concentration of whole liver.

600 gm. of either of these fractions are dissolved in 300 cc. of hot water. The heavy viscous fluid is then extracted four times with *n*-butanol, a total volume of 5 liters being used for the extractions. It is essential that the liver fractions be homogeneously dissolved in the water, since extraction of the dry powder yields a concentrate with very little activity. The wet butanol extracts are clear and deep brown in color. The residue may be discarded. The combined butanol extracts are then concentrated *in vacuo;* small amounts of water should be added to the flask from time to time to keep the solids in solution and to insure the removal of the last traces of butanol. The aqueous concentrate after removal of the butanol is then made up to 1200 cc. 30 gm. of English fullers' earth are now added and the preparation is made strongly acid to Congo red with 6 N HCl. The material is shaken for ½ hour on a mechanical shaker and then filtered with suction. The filtrate is then treated twice more in the same manner with 30 gm. portions of the fullers' earth. The fullers' earth adsorbates containing the riboflavin are then combined and washed with 1 liter of 0.01 N HCl. The washings are combined with the fullers' earth filtrate and the whole is concentrated *in vacuo* to a volume of 300 cc. It is then made alkaline to phenolphthalein with NaOH, and the white amorphous precipitate which forms is filtered off and discarded. A layer of the filtrate 0.5 inch deep is then irradiated in an ice-cooled dish for 3 hours with the light from a frosted 150 watt bulb. The irradiated filtrate is then adjusted to pH 4 to 5 with HCl and stored under toluene in the refrigerator.

The efficiency of this procedure for the removal and destruction of riboflavin from liver extract is attested by the fact that one of the original liver extracts (Fraction B) contained 375 γ of riboflavin per gm. when assayed by the microbiological method of

Snell and Strong (16), while the final concentrates contain less than 0.05 γ per gm. of the starting material when assayed by the same procedure. Since the liver concentrate is fed at a level of 4 per cent in the ration, it will be apparent that the experimental animals receive less than 0.2 γ of riboflavin per 100 gm. of ration from this source.

Assay Procedure—For a reliable assay it is desirable to establish a calibration curve with pure riboflavin along with the material to be assayed. Young male albino rats weighing 40 to 50 gm. are fed the deficient basal ration *ad libitum* until a weight plateau is reached. This depletion period ranges from 3 to 5 weeks. When all of the animals have reached a satisfactory plateau (less than 6 gm. growth in 2 weeks), they are placed in groups of three and are fed the supplements. A satisfactory calibration curve may be obtained when riboflavin is administered at levels of 3, 6, 9, 15, and 30 γ daily. For administration the riboflavin is conveniently made up to a concentration of 30 γ per cc. in 20 per cent alcohol, and is stored in a dark bottle in the refrigerator. The vitamin is pipetted into small individual supplement dishes and is usually consumed immediately by the animals. The unknown samples are usually fed at three levels. Materials high in riboflavin may be diluted by thorough mixing with a small amount of the basal ration. These materials may be weighed accurately into the supplement dishes, and the basal ration is removed until the supplements are consumed.

Results

The growth response to graded doses of riboflavin upon this ration is shown in Table II. It may be seen that the growth increments due to the vitamin are almost linear between 3 and 9 γ. This is the most satisfactory range for the assay of unknown materials. The variation among individual animals in each group is also shown. Despite some variation in the individual rats, there is little overlapping of average daily gains between groups. The closely correlated responses to added riboflavin make it possible to obtain practical results with as few as three animals to a group.

In the regular assay we use five groups of three rats upon different levels of riboflavin. A typical calibration curve is given

in Fig. 1. It may be seen that at the highest level of riboflavin growth of better than 4 gm. daily is obtained.

Table III gives the riboflavin content of some natural products which we have assayed with our ration. We have compared the microbiological assay of Snell and Strong (16) with our bioassay and find good correspondence. The microbiological assay of

TABLE II

Growth Response of Riboflavin-Deficient Rats to Graded Doses of Flavin

Daily dose of riboflavin	No. of rats	Average daily gain for 3 wks.	Daily gain per γ riboflavin	Variation in average daily gains
γ		*gm.*	*gm.*	*gm.*
3	9	0.89	0.30	0.52–1.66
6	9	1.62	0.28	1.38–2.00
9	9	2.20	0.25	1.85–2.67
15	6	2.96	0.20	2.71–3.37
30	6	4.07	0.14	3.33–4.76

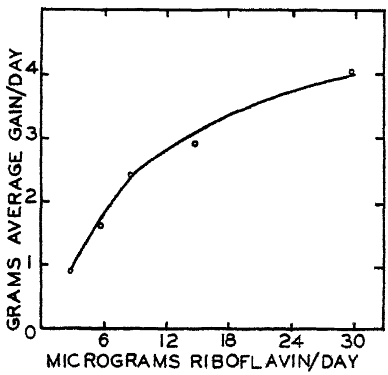

FIG. 1. A typical calibration curve from one series of animals. The riboflavin-free liver extract for these animals was prepared from Fraction B.

riboflavin reported here has been performed according to the direct assay procedure suggested by Snell and Strong (16). In practice, the material to be assayed is finely ground, suspended in water, and autoclaved. A suitable aliquot of the fine suspension is then added to the basal medium for assay. Only with materials having a low riboflavin content on the basis of dry weight is there any considerable discrepancy between the two methods. The

large amounts of solid present in such samples render the direct assay undesirable and better results with the microbiological method are secured if the riboflavin is first extracted from the sample (Feeney and Strong, unpublished data). Contrary to the assumption of El-Sadr *et al.* (10), the animal assay of riboflavin reported by Snell and Strong (16) on skim milk powder has been

TABLE III

Riboflavin Content of Some Natural Products

A comparison of our rat assay method and the microbiological method of Snell and Strong.

Sample	Rat assay, Ration K_{21}	Microbiological assay
	γ *per gm.*	γ *per gm.*
Brewers' yeast	61.5	62.5
Flavin Concentrate 3	2020	2000
" " 6	4000	4000
Liver extract, Fraction B	457	375
Grass 8186	20	23.9
" 1104-9ZB	25	24.1
" 1130-9H	20	24.4
" 2121-6H	22	22.6
Skim milk powder	17	17
Grass 1513-1	10	7.1
" 1513-2	13	11.9
Brewers' grains	2.5	4.5

obtained by our animal assay procedure rather than by the method of Bourquin and Sherman (1).

DISCUSSION

El-Sadr, Macrae, and Work have recently proposed a ration for the assay of riboflavin which contains a charcoal filtrate of liver extract as a source of the vitamin B complex. This ration gives excellent growth when supplemented with riboflavin. However, the utility of this procedure for assay purposes has been demonstrated only with milk. Experience with norit filtrates of liver extract and yeast in this laboratory has shown that variation in the vitamin B_6 and pantothenic acid content of the filtrate may exist, depending upon the kind and amount of norit employed.

Variations in the pH also affect the degree of adsorption of vitamin B_6 and particularly of pantothenic acid upon norit. It seems likely that considerable care must be employed in the choice of conditions for adsorption of riboflavin if the procedure of the English workers is to be employed.

The method which we have described in this publication has been employed with considerable success in the assay of natural products. It is not, however, to be considered a final assay procedure for riboflavin, since later modifications of the ration may yield further improvements. The fullers' earth filtrate of the butanol extract of liver fractions which we employ as a source of the filtrate factors has been shown to be sufficiently low in thiamine, vitamin B_6,[1] and riboflavin to be employed as a source of filtrate factors in the assay of any of these three vitamins. On the other hand, the fullers' earth filtrate is an excellent source of pantothenic acid, factor W, and perhaps other filtrate factors. Rats have been maintained upon this ration supplemented with adequate riboflavin for 8 months without the development of any obvious pathology. Males upon this ration achieve a final weight of approximately 275 gm. and females about 225 gm. Attempts at reproduction on this ration have yielded only partial success, for while some litters have been born, they have never been successfully reared by the mother. It seems likely that the ration is low in vitamin E and perhaps in the lactation factors described by the Japanese workers (17).

SUMMARY

A riboflavin-low basal ration suitable for riboflavin assays with the rat is described. Vitamin B_1, vitamin B_6, and nicotinic acid are supplied in pure form and the remainder of the B complex is supplied in riboflavin-low liver extract. The method of preparing the concentrate is described.

When this ration is supplemented with adequate amounts of riboflavin, normal rates of growth are obtained. With suboptimal amounts of riboflavin, the growth rate is proportionate to the amount of riboflavin fed.

[1] Waisman, H. A., Henderson, L. M., and Elvehjem, C. A., unpublished data.

Our comparison of the results obtained by this method and the microbiological method is given for a number of materials.

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DEFINITION AND ELIMINATION OF CERTAIN ERRORS IN THE HYDROLYSIS, EXTRACTION, AND SPECTRO-CHEMICAL ASSAY OF α- AND β- NEUTRAL URINARY 17-KETOSTEROIDS*

By N. B. TALBOT, ALLAN M. BUTLER, E. A. MacLACHLAN, and R. N. JONES

(From the Department of Pediatrics, Harvard Medical School, and the Infants' and the Children's Hospitals, Boston, and the Converse Memorial Laboratory, Harvard University, Cambridge)

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The purpose of the present paper is to define the potential losses and errors involved in the extraction and colorimetric assay of the urinary neutral 17-ketosteroids ("androgens"). Procedures which largely eliminate these potential inaccuracies are described.

Colorimetric Analysis

A primary requisite for an accurate determination of the concentration of a substance in an unknown solution by colorimetric analysis is that the color developed be qualitatively identical with the color produced by a sample of the same substance in pure solution. In comparing the absorption spectra of the *m*-dinitrobenzene-alcoholic KOH reaction products of crystalline 17-ketosteroids in pure alcohol, on the one hand, and of the neutral fraction of urine extracts in alcohol, on the other, Callow, Callow, and Emmens (1) noted marked differences between the spectra of certain of the latter and of the crystalline hormones below 450 mμ. They were unable to estimate the light absorption at 520 mμ referable to the unidentified chromogenic material and thus to calculate the absorption specifically due to the 17-ketosteroids (see Fig. 1). They, therefore, viewed with suspicion results ob-

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tained on urine extracts showing excessive light absorption at 420 mμ. Furthermore, Langstroth and Talbot (2) have shown that the reaction involving neutral 17-ketosteroids may be appreciably influenced by the presence of such a reactive substance as testosterone, which is a Δ^4 unsaturated 3-ketosteroid. Their experiments furnish specific examples of the fact that the interfering chromogens not only may contribute to the light extinction at 520 mμ but also may interfere with the 17-ketosteroid color reaction.

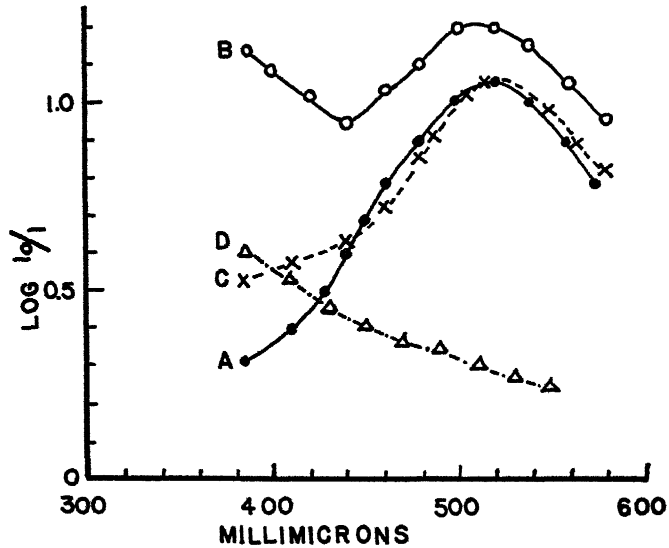

FIG. 1. Extinction-wave-length curves of the colored compounds formed with the *m*-dinitrobenzene-alcoholic KOH reagent by crystalline dehydroisoandrosterone (Curve A), and by the crude fraction (Curve B), the ketonic fraction (Curve C), and the non-ketonic fraction (Curve D) of a representative urine extract.

The experiments that follow were undertaken to define further and to eliminate such errors due to interfering substances in urine extracts.

EXPERIMENTAL

Extinction-Wave-Length Measurements—Fig. 1 presents representative extinction-wave-length curves of the *m*-dinitrobenzene

reaction product obtained respectively with, first, an alcoholic solution of crystalline androsterone (Curve A) and, second, with each of the three fractions of a neutral urine extract described by us in a previous communication (3); namely, the crude fraction (Curve B), the ketonic fraction (Curve C), and the non-ketonic fraction (Curve D). As has been shown previously (4), crystalline 17-ketosteroids (Curve A) have a characteristic curve with a maximum at 520 and a minimum at 380 mμ. Inspection of Curve B reveals that the color formed by the crude urine extract differs definitely from that of Curve A. Instead of a continuous decline in light absorption from 520 to 380 mμ, there is a decline only to 440 and an excessive extinction at 380 mμ. On the other hand, the curve representing the ketonic fraction (Curve C) corresponds much more closely to that given by androsterone (Curve A). Finally, the curve of the non-ketonic fraction (Curve D) shows an appreciable and increasing rise in light absorption from 560 to 380 mμ. This corresponds with the absorption noted in the crude fraction (Curve B) over the range from 440 to 380 mμ.

These spectroscopic analyses of the colors produced in the *m*-dinitrobenzene reaction by a crystalline 17-ketosteroid and by the crude, the ketonic, and the non-ketonic fractions of urine extracts have been extended and confirmed by a series of measurements made with a photoelectric colorimeter. Extinction coefficients[1] were obtained with a green filter (maximum transmission 520 mμ) and a blue filter (maximum transmission 420 mμ). In Fig. 2, the extinction coefficients obtained with the green filter are plotted as ordinates, while those obtained with the blue filter are plotted as abscissae. Line A is drawn through points obtained with various amounts of crystalline androsterone. Line B represents the mean for points obtained with the ketonic fractions of urine extracts. Because of the wide scatter, no line could be drawn through the points which represent the crude fractions of urine extracts. On the other hand, the points given by the non-ketonic fractions clearly describe Line C.

The data of Fig. 2 show that the highest ratio (2.0) for E_G:E_B was obtained with pure solutions of crystalline 17-ketosteroids

[1] E_G and E_B represent the extinction coefficients obtained with the green and blue filters, respectively. The extinction coefficient is the log of the blank galvanometer reading minus the log of the test galvanometer reading.

(Line A). The ratio for the ketonic fractions, represented by Line B, averaged slightly lower (1.8). On the other hand, the average ratio obtained for the non-ketonic fractions (Line C) was much

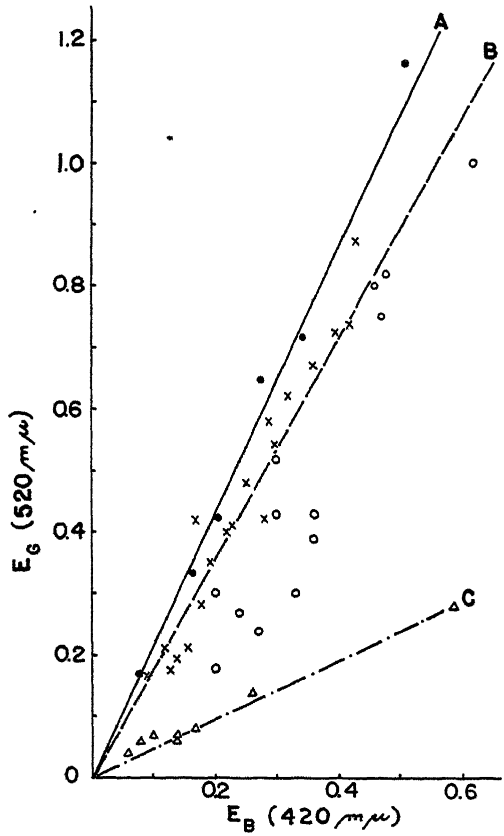

FIG. 2. Extinction coefficients obtained on the colored compounds formed with the *m*-dinitrobenzene-alcoholic KOH reagent by crystalline dehydroisoandrosterone, androsterone, and isoandrosterone (Line A), the ketonic fractions of various urine extracts (Line B), and the non-ketonic fractions of the same urine extracts (Line C). The ordinates represent the extinction coefficients obtained with a green filter; the abscissae represent the extinction coefficients obtained with a blue filter. O represents points obtained for the crude neutral urine extracts.

lower (0.48) than that obtained for either the ketonic or crystalline 17-ketosteroid solutions. As would be expected, the points representing the crude fractions are scattered between Lines B and C.

The foregoing extinction-wave-length measurements of the colors given by the various solutions in the *m*-dinitrobenzene reaction lead to the following conclusions.

The colors given by solutions of the crude fractions are variable. This variability is apparently due largely to non-ketonic substances which react with *m*-dinitrobenzene to give a color which differs from the characteristic 17-ketosteroid color. Therefore, it is to be expected that colorimetric assays made on the crude fraction will be subject to a variable error. It will not be feasible to devise a reliable method for correcting for the errors referable to interfering chromogens in crude extracts until they and their behavior in the *m*-dinitrobenzene-alcoholic KOH reaction have been further defined.

The colors given by solutions of the ketonic fractions approximate those given by pure solutions of crystalline 17-ketosteroids much more consistently than do solutions of the crude neutral extract. Therefore, the colorimetric assay should be more reliable when made upon the partially purified ketonic fraction.

The fact that the colors given by solutions of the ketonic fraction are not always identical with the colors given by crystalline 17-ketosteroids suggests that the ketonic fraction contains either traces of non-ketonic chromogens or ketosteroids other than 17-ketosteroids. These other ketosteroids may, as shown by Callow *et al.* (1) and McCullagh, Schneider, and Emery (5), give colors in the *m*-dinitrobenzene reaction that differ significantly from the colors given by the 17-ketosteroids.

Quantitative Accuracy—Previously published data (3) showed that known amounts of androsterone, dehydroisoandrosterone, and mixtures of the two ranging from 0.05 to 0.30 mg. per sample analyzed could be assayed with an error not exceeding ± 6 per cent. Subsequently in a series of eight determinations of the 17-ketosteroid content of a single urine extract, in which the samples analyzed varied from 0.05 to 0.2 cc., the variation was ± 3 per cent.

In order to determine the consistency with which the α-17-ketosteroids[2] could be determined, 4 aliquots of a single urine sample

[2] In this paper the term β fraction refers to the 3-β-hydroxyketosteroids which are precipitated with digitonin. For brevity and convenience the term α fraction refers to the remaining ketosteroids which are not precipitated with digitonin.

were precipitated with digitonin and the supernatant solution was assayed for α-17-ketosteroids. The results showed a variation of ± 7 per cent from the mean.

Previously published data show the average recovery of known amounts of androsterone, dehydroisoandrosterone (3), and estrone (6) in the ketonic fraction after treatment with Girard's Reagent T to be 93 per cent. The poorest recovery was 85 per cent.

Comparison of Results Obtained with Crude and with Purified Extracts—Table I gives data on the 24 hour excretion of total

TABLE I

Colorimetric Assay of Crude and Purified Extracts

Urine extract No.	Neutral 17-ketosteroids, mg. per 24 hrs.				
	Crude (*a*)	Corrected crude (*b*)	Ketonic (*c*)	(*c*):(*b*)	$E_G:E_B$ of (*a*)
1	1.1	1.0	0.7	0.70	0.83
2	1.5	1.3	0.5	0.38	0.90
3	3.1	2.9	2.1	0.72	1.08
4	4.1	3.8	2.6	0.69	1.00
5	7.8	7.4	6.2	0.84	1.50
6	27.4	27.4	20.0	0.73	1.43
7	5.4	5.2	5.0	0.96	1.50
8	6.4	6.2	5.4	0.87	1.57
9	8.1	7.8	8.0	1.03	1.70
10	13.4	13.0	11.4	0.88	1.71
11	27.0	27.0	26.6	0.99	1.83
12	27.7	27.7	30.0	1.08	1.83

neutral 17-ketosteroids, determined on the crude and the ketonic fractions of urine extracts. Columns (*a*) and (*b*) present the values obtained on the crude fractions; column (*b*) includes the correction for crude non-reactive colored substances described in an earlier paper (3); column (*c*) gives the values for the ketonic fractions. Inspection of column (*c*):(*b*) reveals that in the first six urine extracts the differences between the ketonic and the crude values are greater than can be accounted for by probable losses in the Girard's purification procedure and that the correction applied in column (*b*) is not adequate. The ratios of $E_G:E_B$ for the crude solutions of these same extracts indicate a relatively large amount

of non-ketonic chromogens. A similar analysis of the data in Table I pertaining to the remaining six extracts shows that the correction applied in column (*b*) is relatively satisfactory and that the $E_G:E_B$ ratio indicates the presence of relatively small amounts of non-ketonic chromogens. These data, which confirm the findings of Callow *et al.* (1), suggest that when the $E_G:E_B$ ratio is 1.5 or greater the values obtained following the simple correction for non-reactive colors are reasonably satisfactory. For solutions which give an $E_G:E_B$ ratio of less than 1.5, a significant increase in accuracy is attained by separating the ketonic from the bulk of the non-ketonic substances with Girard's Reagent T before the colorimetric determination is made.

Assuming that the value obtained from the colorimetric analysis of the ketonic fraction is approximately correct, the analytical data on urine Extract 2, which contains a low concentration of neutral 17-ketosteroids, show that overestimations by as much as 260 per cent may occur in the assay of the crude fraction. In spite of this high percentage error, the error in the 24 hour excretion is but 0.8 mg. In a similar way the data on urine Extract 6, which contains a relatively high concentration of 17-ketosteroids, show an overestimation of 35 per cent and of 7.4 mg. in a 24 hour excretion.

Hydrolysis and Extraction

Dingemanse *et al.* (7, 8), Peterson, Gallagher, and Koch (9), and Callow *et al.* (10) have presented evidence which shows that some loss of androgenic activity and of neutral 17-ketosteroids may occur during acid hydrolysis of urine prior to extraction. Dingemanse and Laqueur (8) separated the α- and β-steroids with digitonin and assayed the fractions by biological methods. They noted a greater loss of the β than of the α fraction and showed that this loss could be largely prevented by simultaneous hydrolysis and extraction. Workers using independent hydrolyses have not agreed upon an optimal procedure. The experiments that follow present a more detailed definition of the effect of independent hydrolysis and describe an improved method of simultaneous hydrolysis and extraction.

The inflammability of ether and benzene make these two solvents unsuited to routine procedures of continuous hydrolysis.

Moreover, the tendency of the former to form emulsions and the particular toxicity of the latter are further disadvantages in their

TABLE II

Time Required for Essentially Complete Extraction of Neutral 17-Ketosteroids from Urine by Simultaneous Hydrolysis and Extraction Procedure

Colorimetric assays were made on the ketonic fraction.

Experiment No.	Solvent	Method of hydrolysis and extraction	Total time	Total 17-ketosteroids†
			hrs.	*mg. per l.*
1	CCl_4	Independent*	$\frac{3}{4}$	11.8
2	"	Simultaneous	2	4.4
			4	10.0
			6	12.0
3	"	"	6	12.0
			7	12.5
4	$Cl_2C{=}CCl_2$	"	2	12.6
			3	13.4
			4	13.7
5	"	"	3	13.5
			4	13.7

* Control experiment with independent hydrolysis and subsequent extraction.

† The extracts obtained were washed twice with 25 cc. lots of a 10 per cent solution of $Na_2S_2O_4$ in 1.0 N NaOH, twice with 25 cc. lots 1.0 N NaOH, twice with 25 cc. lots of 0.5 N HCl, and three times with 25 cc. lots of water. The neutral residue was treated with Girard's Reagent T (trimethylacethydrazideammonium chloride, Eastman) according to the procedure described previously (3). The colorimetric assays of the total and α-ketosteroids were carried out on the ketonic fraction. Instead of the Girard's reagent-ketosteroid condensation in alcoholic solution as described previously, the condensation may be carried out in the absence of alcohol and in a brief period of time according to Dr. J. K. Wolfe as follows: The neutral residue is thoroughly dried in a glass flask. 0.5 cc. of glacial acetic acid and 200 mg. of Girard's Reagent T are added. The flask is stoppered with tin-foil and the mixture heated for 10 minutes on a boiling water bath. For the rest of the procedure the directions outlined previously are followed. 200 mg. of Girard's Reagent T will take care of 50 mg. of 17-ketosteroid. The final alcoholic extract should be assayed promptly, because it has been noted that these extracts change on standing. This change, which is manifested by a variable but marked decrease in the E_G: E_B ratio is particularly apt to occur in solutions of the α fraction.

use. The present studies on hydrolysis and extraction are limited to the use of carbon tetrachloride and tetrachloroethylene, two

commercially available non-inflammable solvents. Their boiling points of 78° and 121°, respectively, permit raising the temperature of urine to a point where simultaneous hydrolysis and extraction are practical. To provide as efficient extraction as possible with these heavier than water solvents, the extraction apparatus described by Hershberg and Wolfe (11) has been used.[3] They have shown that in this apparatus neutral 17-ketosteroids are almost completely extracted from hydrolyzed urine in ½ hour.

Time Required for Extraction of Neutral 17-Ketosteroids from Urine by Simultaneous Hydrolysis and Extraction Procedure—Table II presents a series of five experiments on one 5 liter lot of pooled urine. The urine was collected with approximately 7 cc. of concentrated HCl per liter as a preservative and was stored in a refrigerator. To each liter aliquot of the final pooled urine 143 cc. of concentrated HCl were added. In Experiment 1 this mixture was boiled under a reflux for 10 minutes and then extracted for ¾ hour with CCl_4. In the other experiments[4] the cold mixture was placed in the continuous extraction apparatus and simultaneously extracted, heated, and hydrolyzed with carbon tetrachloride or tetrachloroethylene for measured periods of time.

The data of Experiment 1 (Table II), which was run as a control, show that 11.8 mg. of 17-ketosteroids were recovered from the 1 liter aliquot which was independently hydrolyzed and extracted. Experiments 2 to 5 indicate that more than 12.0 mg. of 17-ketosteroids were recovered from the other liter aliquots of the same urine by simultaneous hydrolysis and extraction with carbon tetrachloride for 7 hours or with tetrachloroethylene for 3 or 4 hours.

The data also show a satisfactory agreement in results from duplicate analyses (see also Table III).

Comparison of Recovery of Neutral 17-Ketosteroids from Urine after Various Methods of Hydrolysis and Extraction—In keeping with the observations of others, the higher 17-ketosteroid values obtained in the above analyses after simultaneous hydrolysis and extraction suggested that independent hydrolysis might result in destruction of some 17-ketosteroid. Recovery experiments were,

[3] To minimize heat loss from the apparatus, the cylinder which contains the urine was encased in a thick layer of insulating material.

[4] In these 0.7 gm. of stannous chloride was also added to the mixture. Stannous chloride, a mild reducing agent, is not essential but helps to minimize the formation of emulsions.

TABLE III

Comparison of Recovery of Neutral 17-Ketosteroids from Urine after Various Methods of Hydrolysis and Extraction

The data are expressed as mg. per liter of urine, determined by colorimetric assays on the ketonic fraction.

Experiment No.	17-Ketosteroid* added, mg. per liter	Hydrolysis and extraction procedure†	Total 17-ketosteroids		α-17-Ketosteroids		β-17-Ketosteroids	
			Determined	Theoretical‡	Determined	Theoretical‡	Determined	Theoretical‡
1,A	None	I.	5.0		4.2		0.8	
B	"	I. + $SnCl_2$	5.4		4.6		0.8	
C	"	S.	4.6		3.7		0.9	
D	DHA (21)	I.	19.5	26	10.5	4.2	9.0	21.8
E	" (13)	S.	17.0	18	3.6	4.2	13.4	13.8
2,A	None	I.	8.7		8.7		0.0	
B	DHA (22)	"	24.0	30.7	14.0	8.7	10.0	22.0
C	A (25)	"	32.2	33.7	31.1	33.7	1.1	0.0
D	IA (18)	"	26.6	26.7	11.6	8.7	15.0	18.0
E	A (11) + IA (11)	S.	28.0	30.7	17.4	19.7	10.6	11.0
3,A	None	I.	5.5		5.2		0.3	
B	"	S.	6.7		6.5		0.2	
C	DHA (22)	"	27.5	28.1	6.9	5.8	20.6	22.3
D	" (13)	"	17.0	19.1	4.2	5.8	12.8	13.3
4,A	None	I.	2.6		2.4		0.2	
B	"	S.	2.5		2.5		0.0	
C	A(13)	I.	15.5	15.6	15.5	15.5	0.0	0.1
D	IA (11)	"	13.5	13.6	5.0	2.5	8.5	11.0
E	A (5) + IA (14)	S.	23.6	21.6	8.4	7.5	15.2	14.1

* DHA, dehydroisoandrosterone; A, androsterone; IA, isoandrosterone. The figures in parentheses indicate the mg. of 17-ketosteroid added.

† I. indicates that the aliquot was first hydrolyzed by boiling 10 minutes with 143 cc. of concentrated HCl per liter, followed by extraction of the mixture for 45 minutes with CCl_4. I. + $SnCl_2$ indicates that 0.7 gm. of stannous chloride was added to the mixture in addition to the HCl. S. indicates that the aliquot was simultaneously hydrolyzed and extracted with tetrachloroethylene for a period of 3 hours after 0.7 gm. of $SnCl_2$ and 143 cc. of concentrated HCl were added to the liter aliquot.

‡ The theoretical values represent the sum of the average control determinations and the mg. of crystalline steroid added to the aliquot prior to hydrolysis and extraction.

therefore, carried out in which known amounts of crystalline dehydroisoandrosterone, isoandrosterone, or androsterone were

added to aliquots of urine, the α- and β-17-ketosteroid content of which was known. The analytical data from four series of such recovery experiments are given in Table III. The theoretical values represent the sum of the average control values of each urine sample and the mg. of crystalline hormone added to each aliquot. The results of the individual experiments of Table III are summarized in Table IV.

These data show that independent hydrolysis converts approximately 20 per cent of the added crystalline β-hydroxyketosteroids (digitonin-precipitable), dehydroisoandrosterone and isoandrosterone, to ketosteroids which are not precipitated by digitonin. This finding is at least partially explained by the observations of Butenandt *et al.* (12), who noted that hydrochloric acid hydrolysis results in some chlorination of the 3-carbon atom of dehydroisoandrosterone, and of Wolfe[5] who isolated some chlorodehydroisoandrosterone from an extract of human urine which had been hydrolyzed with hydrochloric acid. It is of interest that destruction of the 3-β-hydroxyl group occurred to approximately the same extent in the Δ^5 unsaturated β-steroid, dehydroisoandro-

TABLE IV

Recovery of Hormones Added to Aliquots of Urine

	Experiment No. (Table III)	Hormone added*	Per cent of added hormone recovered as 17-ketosteroids	Per cent of added hormone appearing after digitonin separation			
				α fraction		β fraction	
				Determined	Theoretical	Determined	Theoretical
Independent extraction and hydrolysis	1,D; 2,B	DHA	67	25	0	42	100
	2,D; 4,D	IA	100	20	0	80	100
	2,C; 4,C	A	100	98	100	2	0
Simultaneous extraction and hydrolysis	1,E; 3,C; 3,D	DHA	98	2	0	96	100
	2,E; 4,E	A + IA	104	102	100	102	100

* DHA, dehydroisoandrosterone; IA, isoandrosterone; A, androsterone.

[5] J. K. Wolfe, unpublished observation.

sterone, as in the saturated β-steroid, isoandrosterone. Because the α-steroid, androsterone, is not precipitated by digitonin, we have no information concerning the effect of acid hydrolysis on this hormone. Acid hydrolysis apparently has an additional effect on the carbonyl group at C-17 of dehydroisoandrosterone, as evidenced by a decrease of total color-producing substances (Table III, Experiments 1,D, and 2,B). This change may be dependent upon the Δ^5 unsaturation, as it does not occur in the saturated compounds, isoandrosterone and androsterone.

The data further show that the above effects of hydrolysis are eliminated by simultaneous extraction and hydrolysis. These findings indicate that simultaneous extraction and hydrolysis of urine are essential for the accurate assay of the ketosteroids in urine samples, especially when detailed information is desired.

Although the shorter time required for complete simultaneous hydrolysis and extraction by tetrachloroethylene favors the use of this solvent, the possible deterioration and difficult evaporation of this reagent render it somewhat unsuitable for routine use.

SUMMARY

Assays of the neutral ketosteroid content of urines made by applying the *m*-dinitrobenzene-alcoholic KOH reaction to the crude neutral fraction of urine extracts are subject to a variable error due to interfering non-ketonic chromogenic substances. This error may be eliminated in large part by purifying the crude extracts with Girard's Reagent T. The solution of the partially purified ketonic fraction thus obtained gives colors in the *m*-dinitrobenzene reaction which approximate but do not exactly correspond with the colors given by solutions of crystalline 17-ketosteroids. This discrepancy may be due to either traces of non-ketonic substances or to the presence of neutral ketosteroids other than the 17-ketosteroids.

Hydrolysis of urine with hydrochloric acid may affect the 3-β-hydroxyl group of β-ketosteroids so that these ketosteroids are no longer separable from the α-ketosteroids by digitonin precipitation. An underestimation of the β fraction and an overestimation of the α fraction may, therefore, occur. Such hydrolysis may also affect the carbonyl group at C-17 of Δ^5 unsaturated ketosteroids so that there is a decrease in total color-producing substance in the

m-dinitrobenzene reaction and hence an error of underestimation by this procedure. These effects of hydrolysis are eliminated by the simultaneous hydrolysis and extraction procedure described here.

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THE MICRODETERMINATION OF GOLD IN BIOLOGICAL FLUIDS

By WALTER D. BLOCK and OLIVER H. BUCHANAN

(*From the Rackham Arthritis Research Unit,* Medical School, University of Michigan, Ann Arbor*)

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The increasing interest in the use of gold salts as chemotherapeutic agents in the treatment of certain diseases (1) has made apparent the necessity of an accurate, specific, and sensitive method for the determination of gold in various biological materials. Moreover, the lack of information regarding the metabolism and pharmacology of this element further emphasizes this need. Previous methods have employed either an electrolytic precipitation, a colorimetric reaction, or a titrimetric procedure. The electrolytic methods such as those of Cadwell and Leavell (2) and Lomholt (3) involve too many technical difficulties to be of practical value. The numerous colorimetric methods (4–6) lack both specificity and accuracy. The method of Merejkovsky (6) seems to be specific and is probably the best, but the large blank from the reagent itself and the inadequate directions of the author make it impossible to duplicate the original results. The iodometric titration first employed by Peterson (7) and modified most recently by Hansborg (8) cannot be applied to tissues and blood because the ferric iron present in these substances interferes with the reaction. This difficulty has been overcome by Tukats (9) by a preliminary precipitation of the gold with oxalic acid. However, this preliminary procedure makes it impossible to apply the method to the determination of very small quantities of gold. Pollard (10) determined gold after conversion to the chloride by titration with hydroquinone, using *o*-dianisidine as an indicator. In order to secure quantitative results it was necessary for other

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inorganic salts to be absent. This condition was obtained by a preliminary precipitation of the gold from the original substance by use of a hydrochloric acid solution of tellurium saturated with sulfur trioxide. A modification of this method for use with urine (11) required large volumes of urine and was limited in sensitivity.

Because none of these methods is entirely adequate, it was thought it would be of value to develop an accurate and specific modification of Pollard's (10) procedure for the quantitative determination of gold in small amounts of blood and urine.

Principle of Present Method

The present method involves the determination of gold by the use of the color reaction produced by *o*-dianisidine and auric chloride in a slightly acid solution buffered with potassium bifluoride. This color production follows Beer's law and can be quantitatively measured in an Evelyn photoelectric colorimeter. Preliminary digestion by use of sulfuric acid and hydrogen peroxide is necessary to oxidize all interfering organic substances. Since the color reaction is specific for the chloride or bromide of gold, it is necessary to convert the separated gold to the chloride by aqua regia. All excess sulfuric acid must be previously removed by evaporation to absolute dryness in order to secure a quantitative conversion of gold to the chloride. Likewise after conversion to the chloride, excess aqua regia must be removed because nitrosyl chloride as well as nitric acid produces a color with *o*-dianisidine. Further, the presence of any excess acid will inhibit the production of the final color with gold chloride. Potassium fluoride and hydrochloric acid are then added to the solution to produce the buffered acid solution. In addition the fluoride is necessary to prevent the reaction of any ferric iron present with the *o*-dianisidine (12).

EXPERIMENTAL

Reagents—

Standard gold solution. 153.9 mg. of gold chloride are transferred to a 1000 cc. volumetric flask and diluted to volume with distilled water. 1 cc. of this solution is equivalent to 0.1 mg. of gold. This stock standard is stable for 2 to 3 days.

Potassium fluoride. 10 gm. of this salt are weighed out ac-

curately and transferred to a 100 cc. volumetric flask and diluted to volume with distilled water. After the salt dissolves, the solution is filtered. This solution is stable for 2 days.

Potassium bifluoride (Merck). 0.75 gm. of potassium bifluoride is weighed accurately and transferred quantitatively to a 100 cc. volumetric flask and diluted to volume with distilled water. This solution should be made fresh every 12 hours.

o-Dianisidine. 250 mg. of *o*-dianisidine are weighed accurately, transferred to a 250 cc. volumetric flask, and 150 cc. of water and 1 cc. of concentrated hydrochloric acid are added. The solution is then diluted to volume with distilled water and will remain stable for 1 day.

Hydrochloric acid (1:4). 25 cc. of concentrated hydrochloric acid are transferred to a 100 cc. volumetric flask and diluted to volume with distilled water.

Hydrochloric acid (1:12). 41.5 cc. of concentrated hydrochloric acid are transferred to a 500 cc. volumetric flask and diluted to volume.

Aqua regia. 10 cc. of concentrated nitric acid are added to 30 cc. of concentrated hydrochloric acid. This solution should be made every day.

Procedure

A known amount of urine (10 to 50 cc.) is placed in a Kjeldahl flask[1] which has been graduated to 100 cc. To this flask 3 to 5 cc. of concentrated sulfuric acid are added. A small glass bead and several drops of caprylic alcohol are introduced into the mixture, which is then slowly heated on a Kjeldahl digestion rack until the solution begins to char. The heating is continued for 5 to 8 minutes, after which the flask is cooled and a 30 per cent solution of hydrogen peroxide (superoxol) is added drop by drop until the solution is water-clear. This requires approximately 8 to 10 drops. The solution is then evaporated to complete dryness over a microburner. The sulfuric acid fumes are removed by inserting into the neck of the flask a glass tube which is connected through

[1] 100 cc. Kjeldahl flasks with necks shortened to 5 to 6 cm. are the size used, as, when they are accurately calibrated with a Bureau of Standards 100 cc. pipette, the calibration mark falls in the narrow neck portion of the flask.

a water trap to a water pump. The flask is cooled, 2 cc. of aqua regia are added, and the flask is heated on a microburner until the solution is evaporated to 0.2 to 0.3 cc. The flask is removed and while still warm a slow stream of air is introduced into the flask, which evaporates the remaining acid and moisture. During the heating the flask should be 5 to 6 cm. above the tip of the flame to avoid local overheating which will decompose the gold chloride. After cooling, the precipitated inorganic salts are redissolved by addition of about 75 cc. of distilled water. 0.75 cc. of hydrochloric acid (1:4), 8 cc. of potassium fluoride, and 1 cc. of *o*-dianisidine solution are added to the flask. The volume is made up to 100 cc. with distilled water, the flask stoppered, and the solution thoroughly mixed. The solution is then transferred to a colorimeter absorption tube, filling the tube to within 1 to 2 cm. of the top. After stoppering, the pink color which reaches its maximum intensity within 3 to 10 minutes is read in the Evelyn photoelectric colorimeter with Filter 440 and a center setting on the galvanometer of 76. The stoppering of the graduated Kjeldahl flask and absorption tube is necessary to prevent a slight color development of the reagents in the presence of air.

The same procedure can be applied to blood, with 1 to 5 cc. of plasma which is placed in a micro-Kjeldahl digestion tube that has been graduated to a 15 cc. volume. The initial digestion requires 1 cc. of sulfuric acid, and the conversion to the chloride is accomplished by 0.5 cc. of aqua regia. The color is then developed by the addition of 0.1 cc. of hydrochloric acid (1:12), 1 cc. of potassium bifluoride, and 0.4 cc. of *o*-dianisidine. This is then diluted to 15 cc. volume and the color read as in the case of the urine. Stoppering of the absorption tube is unnecessary because the potassium bifluoride prevents the production of any color of the reagents in the presence of air.

Results Obtained in Tests with Pure Solutions of Gold Chloride

A series of determinations was conducted with varying quantities of gold ranging from 5 to 300 γ. The results are presented in Table I. When the galvanometer was adjusted to 100 with a stoppered tube containing an aliquot of the blank solution made up of 3 cc. of hydrochloric acid (1:4), 8 cc. of potassium fluoride, and 1 cc. of *o*-dianisidine diluted to 100 cc. volume, a center setting of 76[1]

was obtained as compared to 76^0 when water alone was used. The ratio of photometric density (*L* values) to the concentration of the test solution is a constant (*K*, Table I). Reproducibility of results in these tests was excellent. Below the quantity of 5 γ of gold there is a significant deviation from the *K* value. However, this can be overcome in unknowns by using sufficiently large

TABLE I

Reproducibility of Chemical Method for Determination of Gold Chloride and Constancy of Ratio of Photometric Density to Concentration of Compound

Gold present	No. of tests	Range of galvanometer reading*	Average photometric density† (*L* values)	Calculated *K* value‡
γ				
5	3	96^2, 96^3	0.0155	0.0031
10	4	93^2, 93^3	0.0281	0.0030
15	5	89^3, 90^0	0.0470	0.0031
20	5	87^0, 87^1	0.0605	0.0030
25	5	84^0, 84^2	0.0757	0.0030
30	2	81^0, 81^2	0.0915	0.0031
50	4	69^2, 70^0	0.1580	0.0032
100	4	48^0, 48^2	0.3190	0.0032
150	3	32^3, 33^0	0.4850	0.0032
200	2	22^3, 23^0	0.6430	0.0032
250	3	15^3, 16^0	0.8030	0.0032
300	3	12^0, 12^1	0.9210	0.0031
Average				0.0031

* These values correspond to the maximal pink color produced when taken with a center setting of 76^1.

† This is analogous to optical density as measured on a spectrophotometer and corresponds to the quantity (2 − log 10 of the galvanometer reading).

‡ These values are obtained by dividing the photometric density by the amount of the gold in the test solution.

samples of the substance to give readings in the range of 5 to 300 γ, which was the range desired in urine. For blood, however, the desirable range was 3 to 30 γ. The blank solution for blood which was diluted to 15 cc. contained 0.4 cc. of hydrochloric acid (1:12), 1 cc. of potassium bifluoride, and 0.4 cc. of *o*-dianisidine solution; it gave a center setting of 76^0. The *K* value determined for the range (5 to 30 γ) was constant, averaging 0.020 (0.019 to 0.022).

Recovery of Gold from Urine and Blood Plasma

The results obtained by the addition of known quantities of gold chloride to various volumes of urine and blood plasma are summarized in Table II. In the case of urine, the percentage of recovery varied from 94.2 to 100. The dilution of the urinary digest overcomes any inhibitory effect of inorganic salts on the development of the color as found by Pollard (10). This is well

TABLE II

Recovery of Known Amounts of Gold Chloride Added to Urine and Plasma

Material used	Volume	Gold added	Colorimeter reading	Gold found	
	cc.	γ		γ	*per cent*
Urine	10	0	100^{0}	0	0
"	10	20	86^{3}	19.90	99.5
"	10	50	70^{0}	49.97	99.9
"	10	150	34^{1}	150.00	100.0
"	10	200	25^{0}	194.20	97.1
"	10	250	17^{3}	242.26	96.9
"	50	0	100^{0}	0	0
"	50	10	93^{2}	9.42	94.2
"	50	15	90^{0}	14.77	98.5
"	50	20	86^{3}	19.90	99.5
"	50	25	84^{0}	24.42	97.6
"	50	50	70^{2}	48.97	97.9
"	50	100	49^{0}	100.00	100.0
Plasma	1	0	100^{0}	0	0
"	1	3	89^{0}	2.53	84.2
"	1	5	81^{3}	4.38	87.6
"	1	7	75^{1}	6.18	88.3
"	1	8	71^{1}	7.11	88.9
"	1	10	63^{1}	9.95	99.5
"	1	20	40^{0}	20.00	100.0

illustrated by our results in which the percentage of recovery is approximately the same for 50 cc. of urine as in 10 cc., although the concentration of inorganic salts is 5 times as great in the former. In blood, however, the error was slightly greater, with the range of the percentage recovery being 84.2 to 100. The greatest error in the case of plasma occurs in those instances in which less than 5 γ of gold is determined. For amounts greater than this there

is essentially a 90 per cent recovery. If a greater accuracy than 90 per cent is desirable, a larger volume of plasma may be used.

The specificity of the method in both urine and blood is shown by the colorimeter reading of 100° (Table II) secured for digests of both urine and plasma. This value is identical with that secured with reagents alone. These results indicate that the intensity of the color secured with these materials when they contain gold is due entirely to the presence of the gold and cannot be attributed to the reaction of the dye with any other inorganic salt. This method has been applied to the determination of gold in animal tissues as well as to the study of the metabolism and excretion of various gold compounds.

SUMMARY

An accurate and specific microcolorimetric method for the determination of gold by the use of the Evelyn photoelectric colorimeter has been described. The method is based upon the production of a stable red color by the reaction between auric chloride and *o*-dianisidine in an acid medium in the presence of potassium fluoride. The method is applied to the determination of gold in urine in the range of 5 to 300 γ, and to blood plasma in the range of 5 to 30 γ.

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PHOSPHATIDES AND INORGANIC SALTS

By HALVOR N. CHRISTENSEN AND A. BAIRD HASTINGS

(*From the Department of Biological Chemistry, Harvard Medical School, Boston*)

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To explain the presence of inorganic elements in phosphatide preparations, the existence of combinations between ions and lipids has been suggested by several groups of investigators. Thudichum (1), Koch and Pike (2), and Koch and Todd (3) believed combination could exist between phosphatides and alkali and alkaline earth ions; Peters and Man (4) presented evidence for combination between serum lipids and chloride ions; Rosenbaum and Lavietes (5) between serum lipids and thiocyanate ions. The changes in conductivity and refractive index upon mixing salt solutions and phosphatide suspensions have been held to support the formation of ion-lipid combinations (6). Fabisch (7), on the other hand, found no evidence for binding of chloride in lecithin solutions when using Ag-AgCl electrodes.

In the present communication, we are reporting experimental results of two types which bear on the question of ion-phosphatide combination; namely, (*a*) measurements of chloride, sodium, and potassium ionic activities in aqueous sols of lecithins and cephalins, and (*b*) electrometric titrations of phosphatides in aqueous suspension. The latter experiments will also be examined for the information they provide on the relation between the chemical structure of phosphatides and their behavior towards acids and bases, and the physiological significance of the phosphatides as buffer systems.

The results of these experiments indicate that none of the ions studied is bound by lecithins, but that alkali metal ions are combined with cephalins, increasing in amount with increasing pH. Lecithins and sphingomyelins showed no buffering over wide pH

ranges (approximately pH 3 to 9), while cephalins showed buffering from pH 3 to 11.

EXPERIMENTAL

Isolation of Phosphatides—Lecithins were isolated from egg yolks by Maltaner's modification (8) of the method of Levene and Rolf. A pure white preparation with a nitrogen to phosphorus ratio of 1.00 was obtained. No amino N was detected. From its activity in blood coagulation systems, the cephalin content of this preparation of lecithin was not greater than 0.1 per cent.[1]

Two different procedures were used for the separation of cerebral cephalins: (*a*) that of Levene and Rolf (9), and (*b*), this procedure, modified by precipitating the cephalins from aqueous suspension by dilute hydrochloric acid, according to the method of Renall (10), before their final precipitation from ether by acetone. The preparation first mentioned had a phosphorus content of 4.18 per cent, and a N:P ratio of 0.96; the second preparation, from a different sample of lamb brains, had a phosphorus content of 4.21 per cent and a N:P ratio of 0.92. The advantage of preparations obtained by the second procedure was that a larger part of the inorganic cations was eliminated by the precipitation of the cephalins from water by hydrochloric acid. The Levene and Rolf preparation contained 1.83 per cent K and 0.80 per cent Na; the other preparation, which we shall call Preparation 2, contained 0.29 per cent K and 0.18 per cent Na.

Sphingomyelins were isolated from lamb brains and purified according to the method of Levene (11).

Chloride Ion Activities—The activities of chloride ion in aqueous sols of lecithin and pure NaCl solutions were compared by observing the potentials of the system

$$Ag \mid AgCl \mid Cl^{-}\text{-containing solution} \mid \text{saturated } KCl \mid Hg_2Cl_2 \mid Hg \mid Pt$$

at 38° with a type K potentiometer. The chloride-containing solutions were either standard solutions of known sodium chloride concentration, or lecithin solutions containing known quantities of chloride. The Ag-AgCl electrodes were used for lecithin sols only after they had been carefully tested for constancy and theoretical behavior with respect to their potentials as a function of chloride concentration in standard NaCl solutions.

[1] This estimation was made for us by Dr. Geoffrey Edsall.

Sodium Ion Activities—Measurements were made by a flowing amalgam electrode similar in design to that used by Richards and Conant (12). Amalgams were prepared and handled as described by these authors. Both the amalgam and the solution containing sodium ions flowed rapidly under nitrogen through a 3 ml. chamber during measurements. Potentials were measured at 38° with a type K potentiometer, with a high resistance circuit and a vacuum tube amplifier in the system,

Pt | Hg | sodium amalgam | Na^+-containing solution | saturated KCl agar bridge | saturated KCl | Hg_2Cl_2 | Hg | Pt

The use of a potential-measuring system with negligible current drainage permitted the circuit to be closed throughout the period of measurement without producing polarization at the amalgam electrode. In this way, the steadiness of the potential could be observed and an optimum rate of flow of amalgam established. Potentials were steady within about 0.02 millivolt, reproducible within 0.2 millivolt, and the average of a series reproducible within 0.1 millivolt. Known amounts of sodium chloride and hydroxide were added to sols of lecithins or of cephalins (Preparation 2), and, after 42 hours, the sodium amalgam potentials were measured. The potential of each sol was measured about ten times, and that of each standard NaCl solution an equal number of times. These were interspersed among the measurements of the phosphatide sols. Sodium chloride solutions of reference were selected with concentrations which would give potentials close to those of the phosphatide sols.

Membrane Equilibrium Measurements—Below pH 5, the potentials of amalgam electrodes became highly unstable. Partly to extend the measurements into a more acid region and partly to check our results by a different method, we have made membrane equilibrium studies of the effects of cephalins upon the activities of the ions under consideration. The distribution of ions between aqueous sols of cephalins and a water phase containing no cephalins was studied by analysis of the two phases when equilibrium was established across a cellophane membrane. The Gibbs-Donnan distribution law was used to calculate the active concentrations of ions in the cephalin phase.

The apparatus employed consisted of a cellophane bag with the open end fastened over a short glass tube inserted through a large

rubber stopper (Fig. 1). This was fitted into a 3 × 15 cm. test-tube in such a manner that the cellophane bag and the test-tube formed concentric cylindrical chambers. An aqueous sol of cephalins (Preparation 2), after adjustment to the desired pH by addition of alkali, was introduced into the inner chamber and stoppered. A bubble of nitrogen was included to facilitate mixing. The outside chamber was filled with 0.05 N alkali chloride, and sealed after the inclusion of about 0.5 ml. of nitrogen. The apparatus was attached to a slowly rotating wheel and rotated 48 hours at room

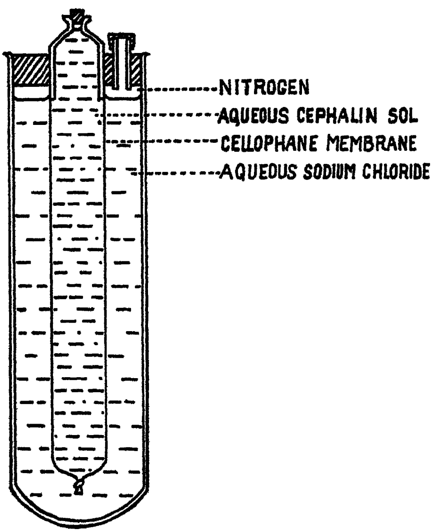

FIG. 1. Apparatus for studying distribution of alkali chloride between cephalin sols and water.

temperature. Movement of the nitrogen bubbles produced effective stirring. At the end of this period, the contents of the two chambers were analyzed for sodium, potassium, chloride, phosphorus, and water. Water was determined by evaporation to constant weight, in high vacuum at room temperature in the case of the cephalin sols. Chloride was determined by the Volhard titration following digestion with nitric acid and silver nitrate; sodium was determined gravimetrically as sodium zinc uranyl acetate, and potassium as the chloroplatinate. These analyses were run in triplicate and checked to 0.5 per cent.

Total P was determined by the method of Fiske and Subbarow. The pH of the contents of each chamber was determined by the glass electrode, standard acetate buffer being used as a standard of reference, and its pH assumed to have the value 4.62. That equilibrium was established in the interval used was shown by experiments in which the final concentrations attained were found to be nearly the same whether the alkali originally had been added to the inside or outside chamber.

Electrometric Titration—Phosphatide sols (about 0.01 M), homogenized in CO_2-free water, were introduced into small test-tubes and portions of standard hydrochloric acid or carbonate-free sodium hydroxide solution added, plus sufficient water to bring

TABLE I

Measurement of Apparent Chloride Activities in Fresh Sols of Varying Lecithin Content

Sol No.	Lecithin	Age of sol	Chloride concentration by analysis	Potential measurement	Apparent Cl ion activity calculated	$\frac{[Cl^-] \text{ calculated}}{[\text{Total Cl}]}$
	gm. per kg. H_2O	*hrs.*	*mole per kg.* H_2O	*mv.*	*mole per kg.* H_2O	
1	0.5	3.5	0.0248	99.9	0.0244	0.98
2	2.4	2.0	0.0240	100.8	0.0235	0.98
3	6.6	2.8	0.0222	102.8	0.0218	0.98
4	14.0	2.5	0.0271	93.8	0.0335	1.24
5	36	3.0	0.0279	92.4	0.0353	1.27
6	36	2.5	0.0510	77.8	0.0555	1.09

all samples to the same final volume. The air above the solutions was replaced by nitrogen, and the tubes were stoppered and shaken. pH values were determined by the glass electrode. In some cases, measurements were made immediately; in others, 24 to 48 hours were allowed to elapse before measurement.

Results

Ionic Activity Measurements—In Table I are presented a series of measurements of Ag-AgCl potentials in lecithin sols of varying lecithin concentrations. The ratio $[Cl^-]/[\text{total Cl}]$ was essentially unity in lecithin sols up to 0.66 per cent. In more concentrated sols (up to 3.6 per cent), the apparent Cl^- activity exceeded the

total molal concentration of chloride. A similar anomalous result was obtained when lecithin sols were allowed to age for several hours. The result of such an experiment is given in Table II. These results are similar to the anomalous behavior of Ag-AgCl electrodes in blood serum previously observed by one of us (A. B. H.).

It may be concluded from these experiments, however, that in fresh dilute lecithin sols no combination occurred between lecithins

TABLE II

Effect of Aging in Apparent Chloride Activities in Lecithin Sols

Sol No.	Lecithin	Age of sol	Cl concentration by analysis	Potential of sol	Apparent Cl ion activity	$\frac{[Cl^-] \text{ calculated}}{[\text{Total Cl}]}$
	gm. per kg. H_2O	*hrs.*	*mole per kg. H_2O*	*mv.*	*mole per kg. H_2O*	
1	2.4	2	0.0240	100.8	0.0235	0.98
2	2.4	5	0.0955	61.9	0.0956	1.00
3	2.4	8	0.0240	98.0	0.0262	1.09
4	2.4	19	0.0240	99.8	0.0247	1.02

TABLE III

Sodium Amalgam Measurements of Na^+ Activity in Cephalin Sols

Concentrations are expressed as mole per kilo of H_2O; concentration of cephalin = 0.010 mole per kilo of H_2O; age of cephalin sol, 42 hours.

Sol No.	Total [Na]	$[Na^+]$	Combined Na	$\frac{\text{Na-cephalin}}{\text{Total cephalin}}$	pH
1	0.0239	0.0221	0.0018	0.18	4.87
2	0.0263	0.0226	0.0037	0.37	7.07
3	0.0287	0.0232	0.0055	0.55	9.13

and chloride ion. Further evidence pointing to the same conclusion will be presented in connection with the titration data.

Three experiments carried out at pH 6.40, 6.85, and 7.21 with sodium amalgam electrodes showed that lecithins had no detectable effect upon the sodium ion activity. In each case, the potential of the lecithin sol fell upon the curve of concentration *versus* E.M.F. for pure NaCl solutions. With cephalin dispersions substantial quantities of sodium were shown to be present in an un-ionized state by measurements with the sodium amalgam electrode, the quantity bound increasing with pH (Table III).

Ionic Distribution Measurements—In the membrane distribution experiments, the Gibbs-Donnan law requires that at equilibrium

$$\frac{[H^+]_i}{[H^+]_o} = \frac{[Na^+]_i}{[Na^+]_o} = \frac{[Cl^-]_o}{[Cl^-]_i}$$

where indicated concentrations denote ionic activities in the phases inside and outside the cellophane membrane. Assuming that Cl is not combined with cephalins, the $[Na^+]$ inside was calculated from the relation

$$[Na^+]_i = [\text{total Na}]_o \times \frac{[\text{total Cl}]_o}{[\text{total Cl}]_i}$$

TABLE IV

Distribution of Na and Cl between Cephalin Sols and Aqueous NaCl

Concentrations are expressed in mole per kilo of H_2O.

Experiment No.		pH	Determined total Na	Determined [Cl]	Calculated $[Na^+]$	Calculated Na-cephalin	Total cephalin	$\frac{\text{Na-cephalin}}{\text{Total cephalin}}$
1	Outside	3.05	0.0488	0.0522				
	Inside	3.05	0.0555	0.0501	0.0509	0.0047	0.037	0.13
2	Outside	6.03	0.0536	0.0538				
	Inside	6.03	0.0680	0.0530	0.0544	0.0136	0.030	0.45
3	Outside	7.01	0.0513	0.0514				
	Inside	6.99	0.0731	0.0499	0.0529	0.0202	0.035	0.58

The sodium bound by cephalins [Na-Ceph] was estimated as the difference between total sodium inside and $[Na^+]_i$. Two sets of experiments showing the combination between cephalin and sodium and potassium are given in Tables IV and V. These experiments indicate that, at neutrality, approximately 0.6 equivalent of base is combined per mole of cephalin.

Titration Measurements—The electrometric titrations of lecithin, sphingomyelin, and cephalin preparations are represented in Figs. 2 to 5. The curves are apparent α curves, constructed from the titration curves by correcting below pH 5 for the chloride ion concentrations equivalent to the hydrogen ion concentrations, and above pH 9 for the sodium ion concentration equivalent to the hydroxyl ion concentrations. The concentrations of hydrogen and hydroxyl ions were calculated from the activity coefficients

given by Lewis and Randall (13) and the values of K_w listed by Clark (14).

TABLE V

Distribution of K and Cl between Cephalin Sols and Aqueous KCl

Concentrations are expressed in mole per kilo of H_2O.

Experiment No.		pH	Determined total K	Determined [Cl]	Calculated $[K^+]$	Calculated K-cephalin	Total cephalin	K-cephalin / Total cephalin
1	Outside	4.24	0.0516	0.0515				
	Inside	4.24	0.0614	0.0516	0.0515	0.0099	0.0295	0.33
2	Outside	7.70	0.0522	0.0515				
	Inside		0.0700	0.0507	0.0526	0.0174	0.0275	0.63

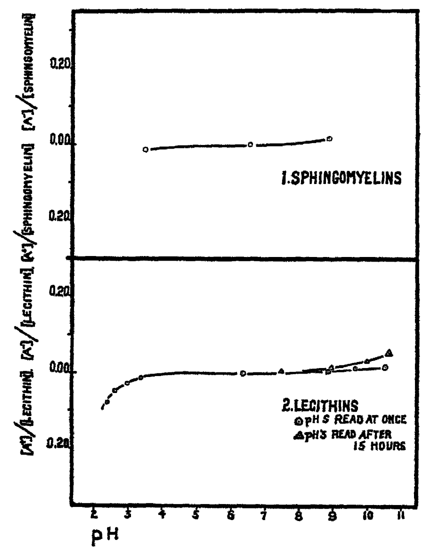

FIG. 2. α curves for the titration of aqueous lecithins and sphingomyelins. $[A^-]$ and $[A^+]$ are the calculated molar concentrations of charged phosphatide ions.

Titrations of lecithins and sphingomyelins showed the absence of any buffering action over wide ranges, in agreement with the assigned chemical structures and in confirmation of our conclusion that neither chloride nor sodium was bound by lecithins (Fig. 2). Above pH 10, some cleavage of lecithins appeared to take place,

as indicated by an increase in buffering activity upon standing 15 hours. At lower pH, the solutions came quickly to equilibrium. The small portion of the curve representing the dissociation of the acidic component of lecithins indicated an apparent pK in aqueous sols of 1.3.

If pH determinations were made at once or a few minutes after alkali addition, aqueous cephalin sols showed only moderate buffer-

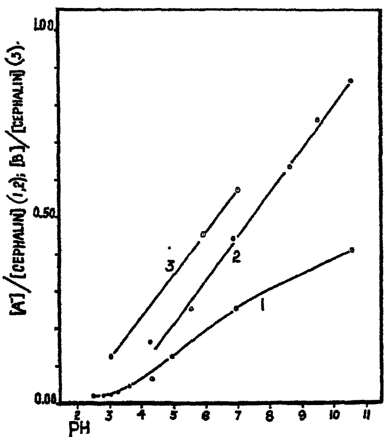

FIG. 3

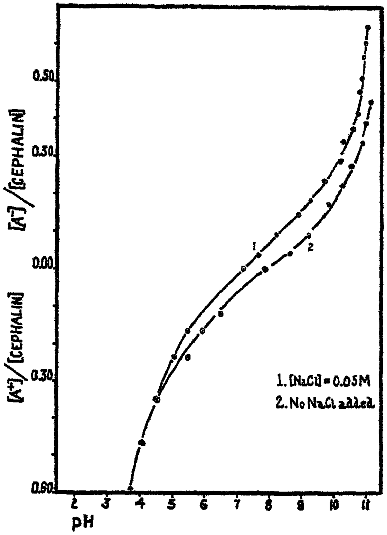

FIG. 4

FIG. 3. The titration of cephalin in aqueous solution (Preparation 2). Curve 1, α curve, pH readings after 3 minutes; Curve 2, α curve, after 42 hour interval; Curve 3, Na bound by cephalin, $[Na^+] = 0.05$ M (Table IV).

FIG. 4. The titration of cephalin prepared according to Levene and Rolf.

ing, comparable with that observed by Fabisch (7) for a crude preparation, and with that observed by Bull and Frampton (15) for cephalin-lecithin mixtures. However, the potentials decreased rapidly at first, then more slowly, finally reaching an apparent equilibrium in about 12 hours. Both preparations of cephalin at equilibrium showed apparent buffering between pH 3.5 and 10.5 (Figs. 3 and 4). The buffer value $\Delta B/\Delta pH$ per mole of cephalin was approximately 0.14 in the physiological pH range.

The effect of the presence of NaCl (0.05 M) on the titration curve of cephalin sols is shown in Fig. 5. It will be seen that the curve is parallel to that obtained in the absence of NaCl, but is displaced to the left. In Fig. 3, the position of the curve (No. 3) relating the amount of sodium bound by cephalins to the pH, compared with the titration curve of cephalins in the absence of NaCl, may be explained in the same manner; namely, that the presence of an increased concentration of Na^+ favored the formation of more Na-cephalin.

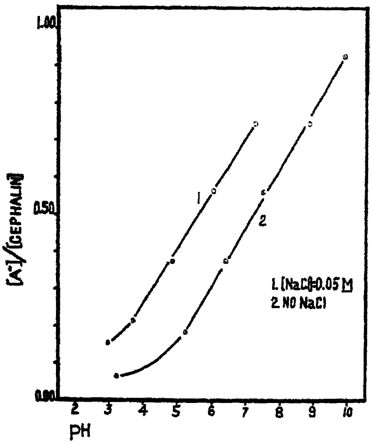

FIG. 5

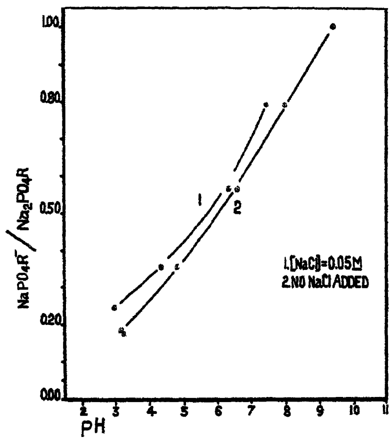

FIG. 6

FIG. 5. The titration of cephalin in the presence and absence of sodium chloride.

FIG. 6. The titration of aqueous sodium α-palmitoyl-β-glycerophosphate.

The buffering of cephalins was not dependent upon the base used to titrate them. Titration with ammonium hydroxide gave the same titration curve as with sodium hydroxide up to pH 7.0.

The anionic behavior of cephalins at all pH values above 3.5 or 4 stimulated us to compare this behavior with that of phosphatidic acids. Sodium α-palmitoyl-β-glycerophosphate was synthesized according to the method described for the stearic ester by Arnold (16). The α curve (Fig. 6) shows a distribution of buffering similar to that shown by cephalins and the pH has a similar sensitivity to added sodium chloride. The similarity between the

titration curves of this compound and cephalins leads to the conclusion that a nitrogenous base such as ethanolamine is not present in a titratable form in the cephalin molecule.

DISCUSSION

The results of investigation by several methods are in agreement in indicating that the only large effect of the three phosphatides upon the activities of the ions studied was that of the cephalins upon cation activities. These results indicate that, at neutrality, cephalins are in combination with 0.5 to 0.6 equivalent of alkali per mole of cephalin. Contrary to the suggestion of Koch and Pike (2), the affinities of cephalins for sodium and potassium were found to be practically identical. The presence of greater quantities of potassium than sodium in cerebral cephalin preparations probably reflects the higher concentration of potassium in the material from which they were extracted.

The results obtained upon the titration of lysocephalins in aqueous solution by Levene, Rolf, and Simms (17) by the addition of 0.9 and 0.95 moles of alkali per mole of phosphorus correspond to our titration data for cephalins, suggesting that these compounds have similar titration behaviors.

Our studies of the cephalins do not support the conception that these phosphatides in aqueous solution exist as zwitter ions of the structure

$$R\cdot O\cdot \underset{O^-}{\overset{O}{P}}\cdot O\cdot CH_2\cdot CH_2\cdot \underset{+}{NH_3}$$

The reasons for this conclusion are (*a*) that at neutrality cephalins contain over 0.5 equivalent of fixed base per mole; (*b*) when these bases have been eliminated or an equivalent amount of acid added, the aqueous solutions are acid. The ease with which the nitrogenous base is lost from cephalins during isolation (Levene and Komatsu (18)) is not in agreement with what is known concerning the stability of the phosphoric ester of aminoethanol, or of the diesters of phosphoric acid (Plimmer and Burch (19)). Both the titration behavior and the instability of cephalins suggest the possibility that cephalins have structures other than those attributed to them.

Our findings indicate that cephalins, in contrast to lecithins and

sphingomyelins, play a rôle in tissue electrolyte distributions, being combined with about 0.5 equivalent of base per mole of cephalin at neutrality and holding this base mainly in an un-ionized form.

SUMMARY

Observations on silver-silver chloride electrodes, amalgam electrodes, on distributions of ions across membranes, and on electrometric titrations indicated (*a*) that lecithins, cephalins, and sphingomyelins do not combine with appreciable quantities of chloride ion, (*b*) that lecithins and sphingomyelins do not combine detectably with sodium ions, over a wide pH range, and (*c*) that cephalin binds sodium and potassium with equal affinity, the amount increasing with pH. The titration behavior of cephalins was shown not to be in good agreement with the structure usually assigned, but much like that of phosphatidic acids. These findings establish for cephalins a possible rôle in biological electrolyte equilibria.

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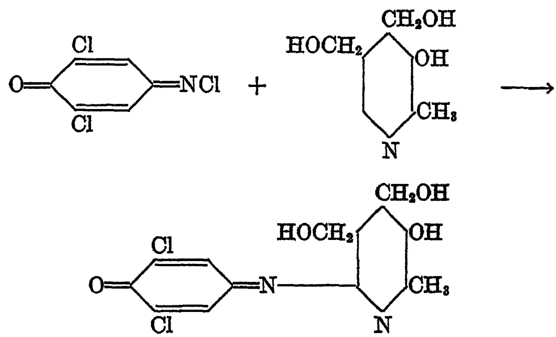
THE FORMATION OF A VITAMIN B_6-BORATE COMPLEX

By JOHN V. SCUDI, W. A. BASTEDO, AND T. J. WEBB

(From the Research Laboratory of Merck and Company, Inc., Rahway, New Jersey)

(Received for publication, July 26, 1940)

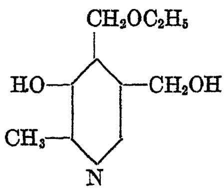
In the course of the development of a colorimetric method for the determination of vitamin B_6 (1) it was observed that 2,6-dichloroquinone chloroimide would condense with the vitamin in the presence of a variety of buffers to give the desired blue indophenol, in accordance with the reaction,



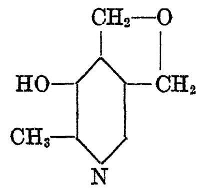
This reaction, however, does not occur in the presence of a borate buffer. Any substance which gives the indophenol reaction in the presence of this buffer is, therefore, not the vitamin. This observation is important to the specificity of the test, since phenols normally react with quinone chloroimides in the presence of borate buffers. Further, 0.001 per cent of borate causes an error in the performance of the test, and borates must thus be considered interfering substances. For these and other reasons, which are discussed subsequently, the nature of the borate interference was investigated.

The polyhydric nature of the vitamin suggested complex formation, involving the vitamin and borate ion in such manner that

the phenolic nature of the vitamin was masked. It was assumed that this combination probably involved the 3-hydroxy and 4-hydroxymethyl groups of the vitamin. In agreement with this assumption, it was observed that compounds (I) and (II) (kindly furnished by Dr. Harris of these laboratories) would condense with the chloroimide reagent in the presence of a borate buffer. Thus the significance of the hydrogen in the 4-hydroxymethyl group was established.



(I)



(II)

That the phenolic hydrogen is also involved in the complex may be inferred from the work of Boeseken (2). Moreover, this involvement was demonstrated directly by comparison of the ultraviolet absorption spectrum of the complex and that of the vitamin.

In the paper of Stiller, Keresztesy, and Stevens, on the structure of vitamin B_6 (3), it was shown that the vitamin in aqueous solution at values of pH 3 or less exhibits a single maximum at 2920 Å. This band is depressed and two new bands appear at 2550 Å. and 3250 Å. as the pH is raised to 7.45 (Fig. 1). When the 3-hydroxyl hydrogen atom is substituted, as for example in the 3-methoxy derivative of the vitamin, the single "acid" band at 2800 Å. persists with increased pH (3). Harris, Webb, and Folkers (4) have attributed the shifts in these bands of vitamin B_6 to structural changes involving the phenolic hydrogen and the basic ring nitrogen.

It is possible to eliminate this shift in absorption by merely adding borate ion to solutions of the vitamin. The absorption of the vitamin in the presence of a borate buffer at pH 7.5 is shown in Fig. 2. The single band at 2920 Å., normally associated with acid rather than neutral solutions, is observed. This behavior would seem to indicate conclusively that the phenolic hydrogen is involved in the borate-vitamin B_6 complex.

In other borate complexes of the Boeseken type the acidity of the boric acid is markedly increased, and it was found that the vitamin B_6-borate complex showed a similar increase. Fig. 3 shows the results obtained in titrations[1] with alkali of vitamin B_6 hydrochloride, boric acid, and a mixture of both substances.

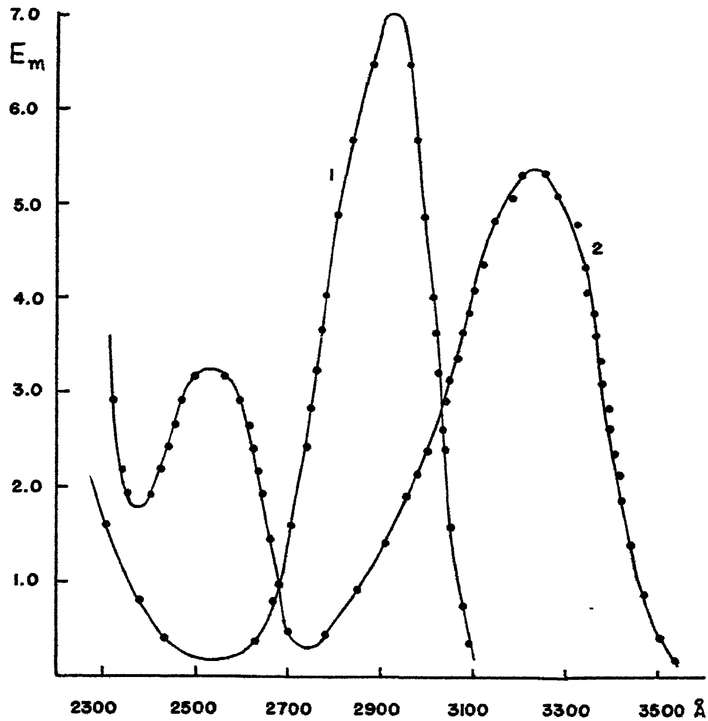


FIG. 1. Curve 1 shows the ultraviolet absorption of the vitamin in an aqueous solution of pH 2.1. Curve 2 is the same at pH 7.5. E_m is the millimolecular extinction coefficient.

Vitamin B_6 neutralizes 2 equivalents of alkali, the mid-points of the two stages occurring respectively at pH 4.72 and 8.96, with corresponding acid ionization constants of 1.9×10^{-5} and 1.1×10^{-9}. The mid-point of the neutralization of the 1st equivalent of alkali by boric acid occurs at pH 9.4, indicating the ionization

[1] The glass electrode was used in these titrations. Values above pH 9.5 have not been corrected.

constant of 4×10^{-10}, in essential agreement with the range of values to be found in the literature for this substance.

In the case of the vitamin-borate mixture the curve shows the expected marked increase in acidity ($K_a = 3.5 \times 10^{-3}$). A similar increase in acidity was not observed upon titration of boric acid in the presence of the 3-methoxy derivative of the vitamin. This is additional evidence for the linkage of boron in the original complex through the oxygen atom in the 3 position and elimi-

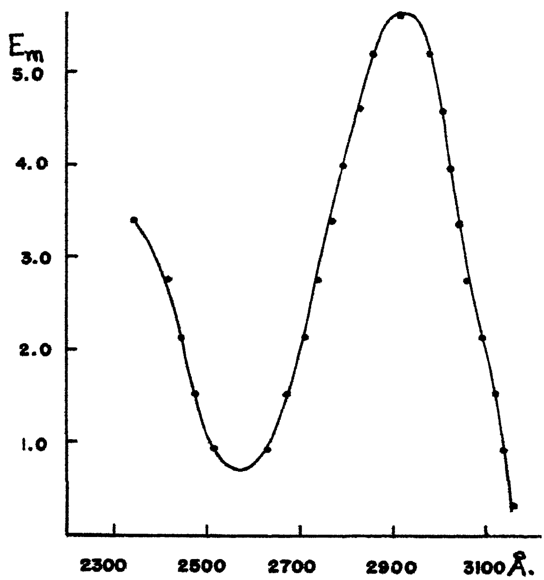
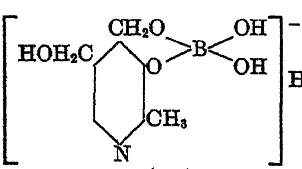


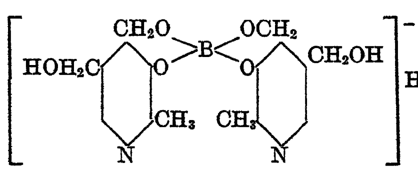
FIG. 2. Ultraviolet absorption of vitamin B_6 in a borate buffer at pH 7.5

nates the possibility that the pair of hydroxyl groups involved is in the 4,5 position.

While a simple increase in acidity does not differentiate between the possible structures ((III) and (IV)), further consideration of the titration data establishes structure (IV).



(III)



(IV)

The data obtained by the potentiometric titration of a mixture of 1.00 mole of boric acid to 1.425 moles of the vitamin hydro-

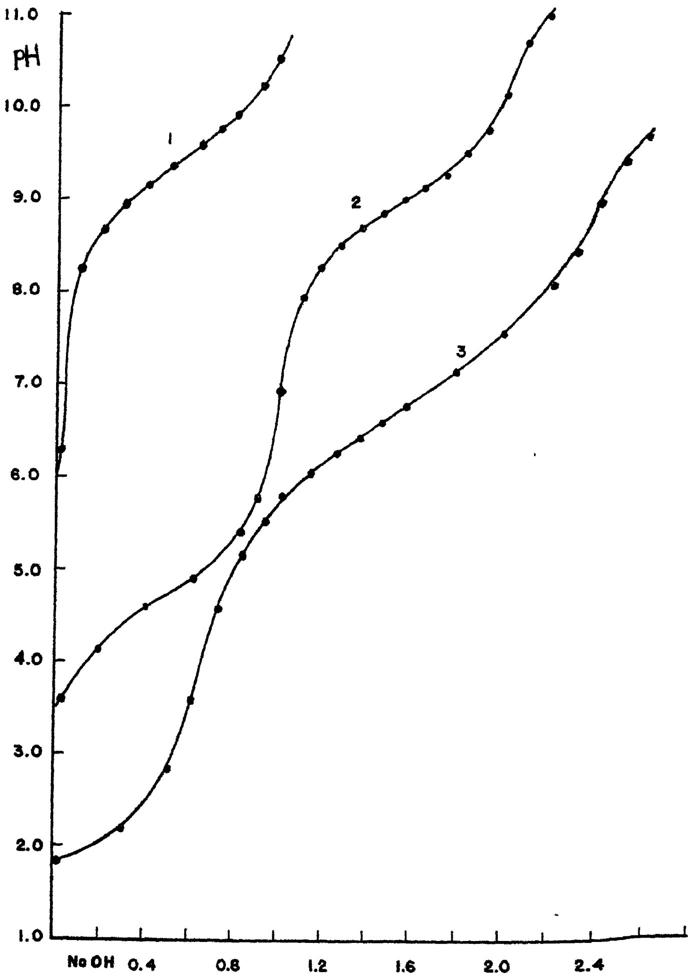


FIG. 3. Potentiometric titration of (Curve 1) boric acid (1 mM), (Curve 2) vitamin B_6 hydrochloride (1 mM), (Curve 3) vitamin B_6 hydrochloride (1.43 mM) and boric acid (1 mM), NaOH in mM.

chloride are shown in Fig. 4. Curve 2 is a differential curve. The high sharp maximum, indicating completion of a titration stage,

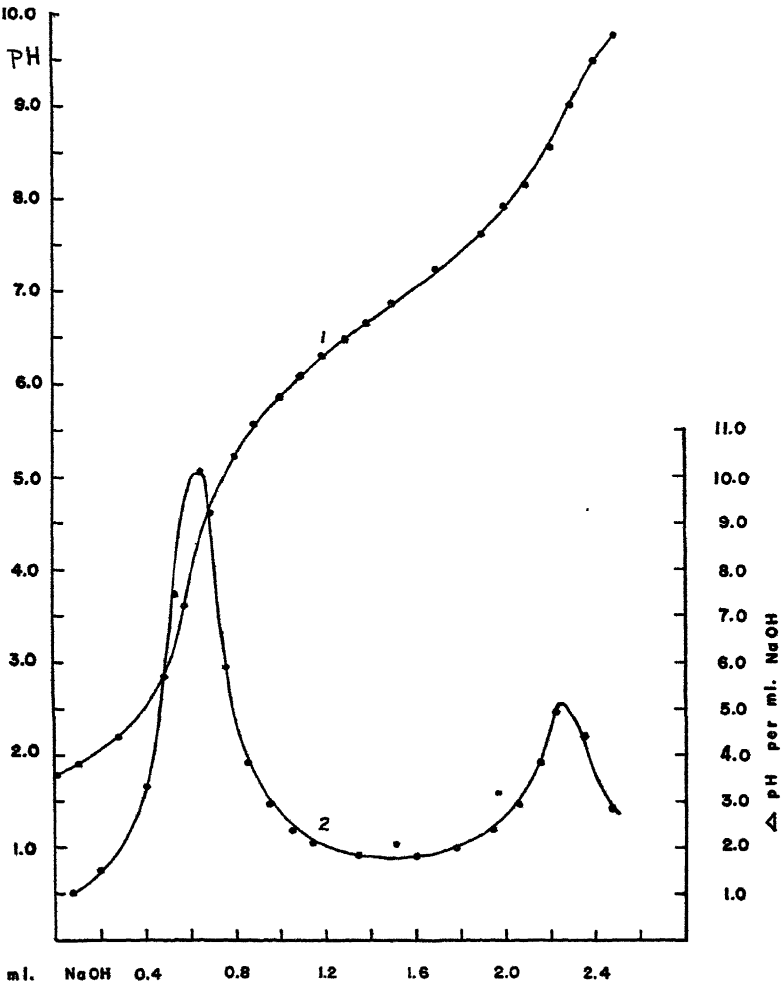


FIG. 4. Potentiometric titration (Curve 1) of 1.00 of boric acid and 1.425 mM of vitamin B_6 hydrochloride. Curve 2 is a differential curve (ordinate to the right).

corresponds exactly to one-half of the vitamin.[2] This relationship indicates that 2 molecules of the vitamin have combined

[2] The small secondary peak shown in the differential curve corresponds to the binding of the 2nd equivalent of alkali by vitamin B_6 hydrochloride.

with 1 molecule of boric acid (present here in excess) to give the more strongly acidic complex. Table I shows the result of varying the boric acid-vitamin ratio in these titrations. The "calculated millimoles of complex" are calculated on the basis of 2 molecules of vitamin to 1 molecule of boric acid. They are in good agreement with the number of equivalents found.

Since it has been shown that the vitamin-borate complex involves 2 molecules of the vitamin to 1 molecule of boric acid, and since the boron atom has been shown, by various means, to be linked through the oxygen atoms in the 3 and 4 positions of the vitamin, structure (IV) is assigned to the complex.

This study is of interest in connection with the parenteral administration of the vitamin. When neutral (pH 7.0 to 7.5) 10 per cent solutions of the vitamin are autoclaved at 20 pounds

TABLE I

Effect of Varying Boric Acid-Vitamin Ratio

Vitamin B_6 hydrochloride	Boric acid	Calculated mM of complex	Alkali used
mM	*mM*		*milliequivalents*
0.496	1.00	0.25	0.24
1.00	1.00	0.50	0.47
1.42	1.00	0.71	0.68
2.00	1.00	1.00	0.98
2.50	1.00	1.00	0.99

pressure for 20 minutes, the solutions become yellow in color and precipitation of a condensation product (4) occurs. From structural considerations, it was conceivable that formation of the borate complex might prevent this reaction. Similar solutions at the same pH, containing 0.5 mole of borate, remained colorless upon autoclaving, and precipitation of the condensation product did not occur.

Dr. Harris kindly furnished us with purified samples of the condensation product. These were shown to possess considerably less activity than an equal weight of vitamin B_6, as shown by the curative assay with vitamin B_6-deficient rats, reported by Unna (5). In additional assays, also performed through the courtesy of Dr. Klaus Unna of the Merck Institute, the borate-containing solutions, after autoclaving, retained their full activity

in the cure of vitamin B_6-deficient rats. It may, therefore, be inferred that the vitamin B_6-borate complex is thermostable, but is completely broken down in the animal organism to liberate the unchanged vitamin.

SUMMARY

Vitamin B_6 has been shown to undergo complex formation with boric acid. Boric acid, with a coordination number of 4, is linked to 2 molecules of the vitamin through the oxygen atoms in the 3 and 4 positions. This complex has the physiological activity of vitamin B_6, and is thermostable in neutral solution.

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CHEMISTRY OF THE CHICK EMBRYO

I. THE DIPEPTIDASE OF CHICK EMBRYO EXTRACTS*

By ALBERT H. PALMER and MILTON LEVY

(*From the Department of Chemistry, New York University College of Medicine, New York*)

(Received for publication, July 24, 1940)

The presence of dipeptidase in the tissues of the early chick embryo has been noted (1). As a part of an investigation of enzymes in relation to embryonic growth, we have found it necessary to devote some attention to the properties of alanylglycine dipeptidase.

Subsequent work will involve the use of the micromethods developed by Linderstrøm-Lang and Holter (2). For the sake of uniformity, the same methods have been employed in the present investigation. Except where otherwise stated, the substrate used was *dl*-alanylglycine.

Preparation of Enzyme Extracts—Eggs of Rhode Island Red hens, laid in trap nests, were used throughout and were received within 48 hours of laying time. The eggs were incubated in a commercial incubator for 72 hours at an average temperature of 39.4° and humidity of 50 to 60 per cent.

A circular section of the egg-shell was cut away by means of a dental separating disk. This can be accomplished without disturbing the shell membrane. The membrane was then cut and the section removed, exposing the embryo. The embryo, together with its extraembryonic vascular system, was separated from the remaining yolk membrane by means of an incision which followed the marginal vein. The incised section was lifted from the yolk by means of forceps and dropped into 0.9 per cent saline. The embryo was stripped of its membranes and extraembryonic

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Presented before the American Society of Biological Chemists at Baltimore (*Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **123**, p. xc (1938)).

vascular system while submerged in saline. We have found the surgical instruments of ophthalmology to be well adapted to this type of dissection. The embryo was washed with saline, freed of adhering moisture, ground with fine acid-washed and ignited sand, and mixed with a measured volume of 30 per cent glycerol. After 2 hours at room temperature, the extraction was continued in the cold room at 5° for 18 to 20 hours. The mixture was shaken at intervals during the extraction. Determination of enzyme activity immediately followed. When necessary, extracts were diluted with 30 per cent glycerol and filtered through cotton immediately before use. We have found that this procedure yields reproducible results and we have used it exclusively. It is necessary to adopt some standard method of preparation because of the instability of the enzyme.

Determination of Enzyme Activity—7.03 c.mm. of 0.2 N *dl*-alanylglycine in 0.07 N NaOH (final pH 7.8), or other solvent to give the desired substrate pH, was pipetted into 8.5 c.mm. of enzyme extract and mixed in microreaction tubes by means of the magnetic stirring device described by Linderstrøm-Lang and Holter (2). The resulting mixture was 0.0905 N with respect to alanylglycine. The tubes were capped and placed in the thermostat at 40° for appropriate intervals. The hydrolysis was stopped by the addition of alcoholic HCl. The extent of hydrolysis was determined by means of the acetone titration method of Linderstrøm-Lang (2). In order to make blank determinations at zero time, alcoholic HCl was mixed with aliquots of the enzyme extract before the addition of the substrate. Individual experimental estimations were carried out in triplicate.

Optimum pH—We found that the pH of optimum activity was 7.8 at 40° and in phosphate buffers. It has been reported that phosphate inhibits the hydrolysis of leucylglycine by malt dipeptidase (3). Phosphate has no inhibitory action on the hydrolysis of alanylglycine by chick embryo dipeptidase.

Kinetics of the Reaction—Within the limits of 20 to 80 per cent hydrolysis the reaction follows the first order equation, $k = 1/t \log a/(a - x)$, where a is the c.mm. of 0.044 N HCl used by complete hydrolysis and x is the actual hydrolysis at time, t, in minutes; k is the first order constant for logs to the base 10. This relationship applies at 30° and 40° and with such substrates as 0.0905

N *dl*-alanylglycine and 0.01132 N *l*(+)-alanylglycine. Typical data are presented in Fig. 1 where the time of hydrolysis is plotted against log $(a - x)$. The straight lines obtained have slopes equal to $-k$ and intercepts equal to log a.

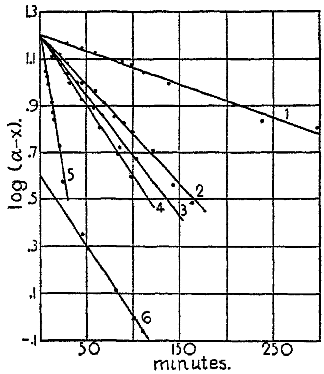

FIG. 1. Log $(a - x)$ is given in terms of c.mm. of 0.044 N alcoholic HCl. Lines 1, 2, and 5 were obtained with 0.0905 N *dl*-alanylglycine at 40°. Lines 3 and 4 were obtained with the same substrate at 30°; Line 6, with 0.01132 N *l*(+)-alanylglycine at 30°.

TABLE I

Relation between Substrate Concentration and Velocity Constant

Temperature 40°; substrate *l*(+)-alanylglycine at pH 7.8. The same enzyme extract was used throughout.

Substrate No.	Concentration of *l*(+)-alanylglycine	First order velocity, $k \times 10^4$	Velocity, c.mm. 0.044 N alcoholic HCl per 100 min.
	N		
1	0.0905	5.8	3.98
2	0.0453	12.9	3.98
3	0.0226	28.0	3.83
4	0.01132	58.5	3.00

It is possible that the entire course of the reaction could be described by means of the first order equation. However, the technical limits of accuracy of the micromethods confine our observations within the above limits.

A study of the relation between substrate concentration and enzyme activity revealed that the numerical value of the first order

velocity constant was dependent upon the substrate concentration, while the absolute velocity was nearly constant over a considerable range. Results are given in Table I. This indicates that we are not dealing with a true unimolecular reaction but with the type discussed by Haldane (4).

A few experiments, in which known amounts of glycine or *l*(+)-alanine were added to the reaction mixture, indicate that the products of hydrolysis strongly inhibit the reaction. This is also in conformity with Haldane's suggestion. Results are included in Table II.

Influence of d(−)-Alanylglycine—This isomer is not hydrolyzed by the enzyme. At least there is no indication of hydrolysis over

TABLE II

Inhibition Due to Reaction Products and to d(−)-Alanylglycine

Temperature 40°; pH 7.8. The same enzyme extract was used throughout.

Substrate No.	Concentration of substrate and inhibitors				Velocity constant, $\times 10^4$	Per cent inhibition
	l(+)-Alanylglycine	*d*(−)-Alanylglycine	*l*(+)-Alanine	Glycine		
	N	*N*	*N*	*N*		
1	0.0453				78	
2		0.0453			No hydrolysis	
3	0.0453	0.0453			45	42
4	0.0453		0.0453		57	27
5				0.0453	55	29

a period of time sufficient to insure complete hydrolysis of the natural isomer. This differs from the findings of Bergmann *et al.* (5) with yeast and intestinal extracts.

The *d*(−) isomer does strongly inhibit the reaction. Experimental results are given in Table II. A comparison of the velocity constants obtained with Substrates 1 and 3 shows that the presence of an equimolecular concentration of *d*(−)-alanylglycine resulted in a 40 per cent inhibition. In terms of the Michaelis theory, this would indicate that the association constants for the formation of the two complexes, enzyme + *d*(−)-alanylglycine and enzyme + *l*(+)-alanylglycine, are of the same order of magnitude. This observation may be of significance in connection with Berg-

mann's (5) theory of specificity in that it indicates that the ability of the enzyme to combine with the substrate is not the sole controlling factor in determining the possibility of hydrolysis of stereoisomers.

Relation between Enzyme Concentration and First Order Velocity Constant—In general the velocity constant of a homogeneous catalytic reaction is expected to be proportional to the concentration of catalyst. In the present case this relationship does not hold. To test the relationship, we used varying volumes of stock extracts

TABLE III

Relation between Activity and Enzyme Concentration Expressed in Terms of Equation Log k = I + S Log V

Extract No.	Substrate	Temperature of experiment	S
		°C.	
1	0.0905 N *dl*-alanylglycine	40	1.46
2	Same	40	1.47
3	"	40	1.31
4	"	40	1.33
5	"	40	1.30
6	"	40	1.36
7	"	40	1.41
8	"	40	1.42
9	"	40	1.35
10	"	40	1.37
11	"	30	1.30
12	"	30	1.32
13	0.01132 N *l*(+)-alanylglycine	30	1.34
Average slope at 40°			1.38

in constant total volumes of reaction mixtures. When the logarithms of the first order velocity constants were plotted against the logarithms of the volumes of extracts used, a straight line was obtained. The intercepts of these lines varied from one stock extract to another, but the slopes were the same within the expected error. In experiments with thirteen independent extracts, and with several independent preparations of alanylglycine, the average slope was found to be 1.38 with extremes of 1.30 to 1.47. The data are given in Table III.

This type of relationship is expressed by the equation $\log k = I + S \log V$, when k is the first order velocity constant, V the c.mm. of stock extract used, and I is the intercept. I is equal to $\log k$ when 1 c.mm. of stock extract is used. The significance residing in the slope, S, is obscure. It may be due to some type of dissociation of the enzyme into inactive components. It is not due to the presence of the $d(-)$ isomer, for the same slope was obtained when $l(+)$-alanylglycine was the substrate. (See Extract 13, Table III.) It is unlikely that it is due to inactivation

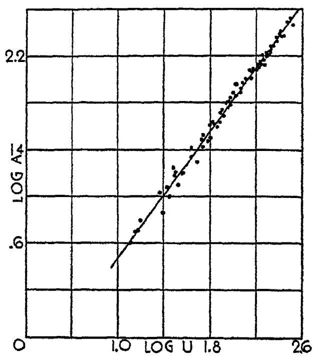

FIG. 2

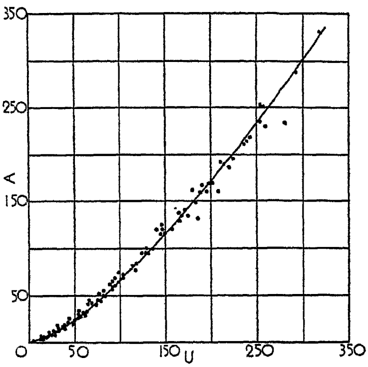

FIG. 3

FIG. 2. Linear relationship between log U and log A where U is the number of units and A the activity per sample as defined in the text. The line is calculated. The points were obtained in ten different dilution experiments.

FIG. 3. Relationship between activity A and amount of enzyme U. The line is calculated. The points were obtained from ten dilution ex periments.

during hydrolysis, for a 10° change in temperature has little, if any, effect, as shown by Extracts 11 and 12 in Table III.

Definition of Enzyme Unit—No completely rational unit of enzyme concentration may be defined, since chick embryo dipeptidase has not yet been obtained in pure form. Our purpose will be as well served by a system of arbitrary units, as long as such a system is consistent with the relationship described in the previous section.

For convenience we multiply the first order velocity constant, k, calculated in minutes and common logarithms, by 10^4 to obtain the activity A. Our standard stock solution gives a first order

velocity constant of 0.03. We then define a unit of enzyme as 1/300 of the amount present in 8.5 c.mm. of this standard solution. All other activities may be converted to units by use of the equation, $\log A = -0.94 + 1.38 \log U$ in which U is the number of units of enzyme corresponding to A.

In Figs. 2 and 3 all the experimental points at 40° summarized in Table III have been converted to this basis so that the uniformity of results may be shown.

Fig. 2 demonstrates the validity of the slope of 1.38 and Fig. 3 shows the direct relation between activity and number of units. A first order velocity constant of 0.03 is near the upper limit at which measurements of reaction velocity can be made with any confidence of accuracy by our methods.

To recapitulate, the definition of the unit is 1/300 of that amount of enzyme which at 40°, in 15.53 c.mm. of solution at pH 7.8 containing 0.0905 N *dl*-alanylglycine and 16.4 per cent glycerol by volume, will give a first order velocity constant of 0.03, with common logarithms and time in minutes.

SUMMARY

The hydrolysis of *l*(+)-alanlyglycine by chick embryo dipeptidase follows the first order reaction equation within the limits of 20 to 80 per cent hydrolysis.

The relationship between enzyme concentration and the unimolecular velocity constant is described by the equation $\log k = I + S \log V$.

d(−)-Alanylglycine strongly inhibits the reaction but is not hydrolyzed by the enzyme and has no influence upon the kinetic type of the reaction.

The reaction is inhibited by the products of hydrolysis.

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CHEMISTRY OF THE CHICK EMBRYO

II. WEIGHT, NITROGEN, AND DIPEPTIDASE ACCUMULATION

By MILTON LEVY and ALBERT H. PALMER

(*From the Department of Chemistry, New York University College of Medicine, New York*)

(Received for publication, July 24, 1940)

The mass of information collected and correlated by Needham in his monograph (7) demonstrates the importance of and interest in chemical embryology, the complexity of the subject, and, in many respects, the incomplete and unsatisfactory character of the available data. The material presented in this paper covers the accumulation of weight, nitrogen, and dipeptidase by the chick embryo in the course of its development from 1.5 to 18 days incubation. While weight and nitrogen data from 4 to 20 days incubation are found in several publications, few data before the 4th day are given and there are no quantitative estimations of the enzyme at any time.

Materials and Methods

The source of eggs, incubation practice, and method used for isolating the embryos have been described (8). The smaller embryos could not be lifted from the yolk by forceps, but when the cut area was touched with a bit of filter paper, they would adhere to the paper. They were then released by shaking in saline. After the extraembryonic membranes and the allantois, when present, were removed, the embryo was transferred to a smooth metal section lifter. Adhering saline was removed with pointed bits of slightly moist filter paper. The moisture tended to prevent sticking of the small embryos. The embryo was then picked up on an arrow-pointed needle and transferred to a suitable tared vessel for weighing. The vessel used depended on the size of the embryo and the use for which it was intended. The largest embryos were dried by rolling on filter paper. This resulted in the loss of some

feathers, but the amount was unimportant. Embryos up to 10 mg. were weighed on a microbalance. Larger ones were weighed on an ordinary analytical balance. All weights are recorded in mg.

The method used for nitrogen estimation varied according to the size of the embryo. Larger embryos were transferred from weighing bottles or stoppered test-tubes to Kjeldahl flasks and digested with Se and Cu catalysts according to standard techniques. If necessary, the digest was diluted and an aliquot taken for distillation. Smaller embryos were weighed in stoppered test-tubes and transferred to 25 × 200 mm. tubes for estimation of the nitrogen by the micromethod previously described (4). The smallest embryos were weighed directly in 13 × 100 mm. test-tubes and the nitrogen was estimated by the decimicromethod (4).

Alanylglycine dipeptidase was estimated in the arbitrary units previously described (8). The embryos up to 2 gm. in weight were ground by use of sand and a glass rod in the vessels in which they were weighed, a suitable volume of 30 per cent glycerol was added, and extraction carried out as described. Larger embryos were transferred to a mortar, cut up with scissors, ground with sand, and extracted with glycerol. In any case the extract was finally diluted so that the activity per sample (8.5 c.mm.) was suitable for a 15 to 30 minute hydrolysis. Hydrolyses were run in triplicate and blanks in duplicate. From the number of units per sample the total dipeptidase per embryo was calculated by dividing by the volume of the sample in c.mm. and multiplying by the total volume of extract (weight of embryo in mg. + volume of 30 per cent glycerol used in c.mm.).

EXPERIMENTAL

The results of the measurements are given in Table I along with the number of individual embryos used at each age. There is considerable variability in the individuals of a given batch of eggs and period of incubation. This is due to a number of factors, such as variable extent of "body heating" in the hen, position in the incubator, and to "biological" variation. A sufficient number of embryos has been used at each age to establish the trend of the data and to stabilize the averages. Each age includes embryos from several different batches of eggs and the data were not obtained seriatim.

TABLE I

Accumulation of Weight, Nitrogen, and Dipeptidase in Rhode Island Red Chick Embryos

Age	Weight	Total N*	N†	Dipeptidase	Dipeptidase†
days	*mg.*	*mg.*	*per cent*	*units* $\times 10^{-3}$	*units per mg.*
1.5	0.72	0.0083 (18)	1.27	1.26 (8)	1450
1.75	1.39			1.90 (8)	1500
1.85	1.45	0.0191 (9)	1.32		
2.02	2.99	0.0277 (18)	1.12	3.58 (15)	995
2.21	4.65	0.0430 (10)	0.92		
2.52	10.40	0.077 (12)	0.86	10.57 (8)	910
2.71	12.8	0.102 (10)	0.82		
2.83	15.3	0.119 (10)	0.78		
3.00	20.0	0.148 (28)	0.79	16.0 (21)	765
3.37	43.6	0.312 (18)	0.72		
3.50	57.4			47 (8)	865
3.75	78.0	0.53 (15)	0.68		
4.04	118	0.83 (9)	0.68	80 (8)	710
4.40	150	1.03 (12)	0.69		
4.50	181			129 (8)	700
4.83	217	1.33 (17)	0.61	155 (8)	680
5.50	349			247 (8)	710
6.00	502	3.19 (26)	0.63	362 (9)	720
7.00	928	6.13 (11)	0.64	660 (8)	730
8.00	1,472	10.7 (10)	0.73	960 (16)	660
8.50	1,814			1,450 (8)	800
9.00	2,170	16.6 (11)	0.74	1,450 (15)	695
10.00	3,170	26.8 (10)	0.79	2,670 (10)	890
11.00	4,180	34.7 (10)	0.85	3,780 (9)	880
12.00	6,110	56.8 (12)	0.95	6,117 (9)	975
13.00	7,960	91.9 (8)	1.13	8,440 (9)	1090
14.00	10,320	162 (20)	1.49	13,700 (8)	1300
15.00	12,900	228 (23)	1.71	18,500 (9)	1490
16.00	16,100	341 (10)	1.99	22,150 (8)	1510
17.00	19,100	405 (11)	2.13	19,500 (15)	1020
18.00	21,900	425 (10)	1.94	17,600 (8)	820

* The parenthetical figures indicate the number of individual embryos used in obtaining each average. The number of embryos used for the average weight is the sum of these figures, in the third and fifth columns.

† The figures in these columns were obtained by averaging the figures for individual embryos rather than by dividing the averages for N or dipeptidase by the average weight.

In Fig. 1 the data are plotted with the ordinates that Murray (5) used for his weight data; *i.e.*, log Q against log A, where Q is used to designate whatever quantity is measured and A is the age in days. This type of plot may be called an accumulation diagram. Each set of data is dissected into a series of straight lines indicating that $d \log Q/d \log A$ for the period covered by each line is constant. Each line is a section of a line described by $\log Q = i_Q + a_Q \log A$, where i_Q is the extrapolated intercept at $\log A = 0$ (1 day) and a_Q is the accumulation coefficient.

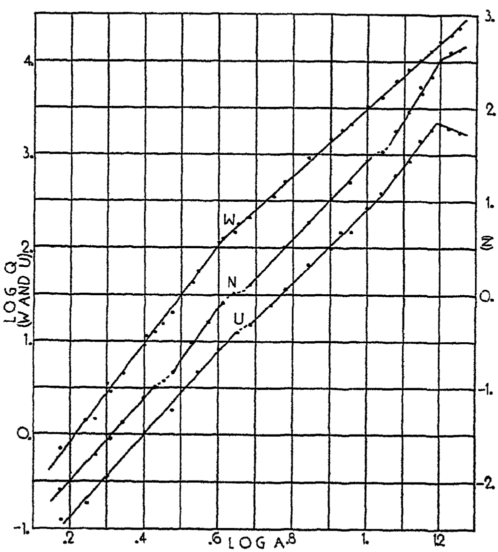

FIG. 1. Log Q (wet weight, W, in mg., nitrogen in mg., and units of dipeptidase, U, per embryo) plotted against log A, the logarithm of the age in days. The units of dipeptidase have been multiplied by 10^{-4} for convenience in plotting.

Each of the straight line sections will be called a phase and identified by the symbol P with superscript and subscript indicating the beginning and end of the phase. Thus $P_{4.5}^{2.5}$ indicates a phase beginning at 2.5 days and ending at 4.5 days. The region of juncture of two phases will be called an interphase. In Table II are collected the phase data. The values of i and a were obtained from large scale plots. For comparison there are included values of a

and i obtained by similar treatment from data found in the literature. The agreement is, in general, good and emphasizes the reality of the phases. Interphases vary between the various sets by approximately 0.5 day where they are comparable. In the

TABLE II

Phases of Growth

Weight

Phase	$P_{4.5}^{(1.5)*}$		$P_{20}^{4.5}$	
Intercept or slope	i_W	a_W	i_W	a_W
From Fig. 1	−1.24	5.5	−0.17	3.61
Murray(5)			−0.19	3.62
Penquite(9)			−0.24	3.62
Schmalhausen (10)†	−1.2	5.1	−0.17	3.61
Byerly(2)†	−0.78	4.6	−0.15	3.62

Nitrogen

Phase	$P_{2.5}^{(1.5)}$		$P_{4.5}^{2.5}$		$P_{10.5}^{4.5}$		$P_{15}^{10.5}$		$P_{(20)}^{15}$	
Intercept or slope	i_N	a_N	i_N	a_N	i_N	a_N	i_N	a_N	i_N	a_N
From Fig. 1	−3	4.6	−3.3	5.3	−2.7	4.1	−5.0	6.2	0.25	1.9
Murray(6)					−2.3	4.1	−4.8	6.0	−0.21	2.3
Penquite(9)					−3.3	4.5	−6.0	7.1	0.63	1.6

Dipeptidase

Phase	$P_{4.5}^{(1.5)}$		$P_{10.5}^{4.5}$		$P_{15}^{10.5}$		$P_{(20)}^{15}$	
Intercept or slope	i_D	a_D	i_D	a_D	i_D	a_D	i_D	a_D
From Fig. 1	2.2	4.4	2.55	3.8	1.0	5.3	9.3	−1.6

* (1.5) indicates the lowest age studied. The interphase ages are approximate.

† Both Schmalhausen's(10) and Byerly's(2) data for $P_{4.5}^{(1.5)}$ consist of very few points. The values of a and i are therefore rough approximations.

earlier phases where 0.5 day is a long biological period, comparable data are not available.

Fig. 2 shows the changes in nitrogen and dipeptidase concentration on a wet weight base during the course of development. The general courses of the curves justify the various phases we have

shown in the accumulation data. Thus comparison of a_W and a_N during $P_{2.5}^{(1.5)}$[1] shows that the per cent N should be falling rapidly, more slowly in the region of the first interphase, and should be nearly constant in $P_{4.5}^{2.5}$ with a slow rise in $P_{10.5}^{4.5}$, and a rapid rise during $P_{15}^{10.5}$ followed by a fall in P_{20}^{15}.

Similar phases can be deduced for dipeptidase. Fig. 1 shows two phases for weight. Accordingly if there was but one phase of dipeptidase accumulation, only two general trends in Fig. 2 should be found. Examination of the curve shows at least four such trends. The drawing of the accumulation diagram in Fig. 1 took this into account. An interphase at 2.5 may be present, but is not clear in our data. An interphase near 4.5 is probably present. Interphases at 10.5 and 15 days are clearly present.

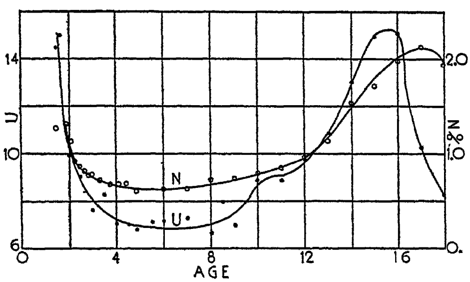

FIG. 2. Curve U, units of dipeptidase per 0.01 mg. of embryo. Curve N, per cent nitrogen. Age is given in days.

DISCUSSION

Growth is defined as the natural increase of a living organism. This increase may be in weight, nitrogen, complexity of form, or function of any other entity upon which we may focus our attention. We may use the terms weight growth, nitrogen growth, morphological growth, etc., to distinguish the category of growth discussed.

Growth rates should be defined in terms of the calculus; *i.e.*, for any growing quantity Q, the growth rate is dQ/dA at the time A. A plot of Q against A will have a slope at any value of A equal to the growth rate. Dividing the growth rate by Q gives

[1] The parentheses indicate that 1.5 is not an established interphase but the age of the youngest embryos studied.

a measure of the multiplication of the organism and we will call it the multiplication rate. It has also been called the relative growth rate or, in terms of per cent, the percentage growth rate. It is equal to $(1/Q)$ (dQ/dA) or $d \ln Q/dA$ and can be obtained from the slope of the curve when $\ln Q$ is plotted against A. This is the curve which Brody (1) maintains is made up of a series of straight lines and which he therefore divides into phases characterized by constant multiplication rates. We have not found the fitting of straight lines to these plots to be satisfactory because, to an undesirable degree, the lines obtained are subjectively determined. In addition, the phases are shorter than on the accumulation diagram.

When the multiplication rate is multiplied by the age, we obtain

$$A \frac{d \ln Q}{dA} = \frac{d \ln Q}{d \ln A} = \frac{d \log Q}{d \log A}$$

which we have called the accumulation coefficient and which is the slope on an accumulation diagram such as Fig. 1.

In a sense the accumulation coefficient is a multiplication rate on a new time scale; namely, a logarithmic one. The logarithmic scale has certain of the properties of "biological time" as related to ordinary time. Thus a unit of ordinary time as measured by a pendulum is independent of the history of the object whose changes in time we are measuring. In contrast equal units of "biological" and logarithmic time scales, as measured by a pendulum, contain many more swings of the pendulum far from the origin (0 for pendulum time, $-\infty$ for logarithmic time) than they do near it and their lengths in pendulum swings therefore depend on the history of the object.

We have defined growth phases as periods during which the accumulation coefficient is constant. Some suggestion of the determining factors in various growth phases can be made. Thus the interphase at 4.5 days very nearly coincides with the time of maximum relative weight of brain compared to body. The weight accumulation prior to this interphase is largely influenced by brain weight; afterwards body growth is the important factor. The large proportion of cephalic fluid in the brain accounts for the decreasing per cent of nitrogen of the whole body during the first phase of weight accumulation and the relative dryness of muscular tissue

accounts for the increasing per cent of N in the last part of Fig. 2. Interpretation of the nitrogen and dipeptidase phases requires more detailed analysis, but we believe that each phase represents the emergence to predominance of the growth of some group of organs. Emergence to predominance should not be taken to mean that growth of the particular group of organs is confined to a single phase. The accumulation of weight in the head of the chick embryo certainly continues after 4.5 days, but its contribution is overshadowed by the contribution of body growth to the weight.

It is interesting to note that the same interphases appear in the several accumulation diagrams. Thus 4.5 days represent an interphase in weight, nitrogen, and dipeptidase, 10.5 days in nitrogen, dipeptidase, dry weight, and ash (Penquite's (9) and Murray's (6) data). The interphase at 15 days is present in all of these also.

Huxley (3) has studied relative growth of various parts of organisms by means of a heterogonic growth equation, which in logarithmic form may be written $\log Q = \log b + k \log Q'$, where Q and Q' are measurements on two different parts, such as body weight and chela weight in the fiddler crab, k is a relative growth coefficient, and $\log b$ is an intercept constant. Phases may appear in plots of $\log Q$ and $\log Q'$, as indicated by abrupt changes in the slopes of the lines. Thus for the relationship mentioned an abrupt change at 1.1 gm. of total weight occurs. Our phase equation is consistent with this type of relationship. Thus, by combining equations for N and weight in the same period, we obtain

$$\log \mathrm{N} = \left(i_{\mathrm{N}} - \frac{a_{\mathrm{N}}}{a_W} i_W \right) + \frac{a_{\mathrm{N}}}{a_W} \log W$$

Phases of heterogonic growth will appear whenever a_{N} and a_W change their ratio.

SUMMARY

The accumulation of weight, nitrogen, and dipeptidase in Rhode Island Red chick embryos has been measured from 1.5 to 18 days of incubation.

The accumulation of any of these quantities can be divided into phases of constant accumulation coefficient, $d \ln Q/d \ln A$.

The times of division between phases often appear on more than one of the accumulation diagrams. The interphase ages probably indicate changes in the predominance of factors determining accumulation.

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THE RELATION OF ZINC TO CARBONIC ANHYDRASE*

By E. HOVE, C. A. ELVEHJEM, and E. B. HART

(*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*)

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Keilin and Mann (1) have reported that the red blood cell enzyme carbonic anhydrase contains 0.31 to 0.34 per cent of zinc, and that all of the zinc within the red blood cell is found in this enzyme. If it could be demonstrated that the presence of zinc is essential to the activity of the enzyme, it would mean that the first direct, isolated, physiological function of zinc has been found. For several years studies have been conducted in this laboratory on the physiology of the zinc-deficient rat. Marked decreases have been observed in pancreatic trypsin and amylase (2) and in intestinal phosphatases (3). Bone phosphatases showed no significant changes. These observations pointed to a disruption in digestive and absorptive processes in the gastrointestinal tract, and were interpreted as secondary effects of the deficiency (2). However, they did explain the decreased rate of absorption and disturbances in nitrogen metabolism previously noted (4).

In this paper we wish to report our results on the purification of carbonic anhydrase and the determination of carbonic anhydrase in the zinc-deficient animal.

EXPERIMENTAL

Method for Determination of Carbonic Anhydrase—The two compartment "boat" technique of Meldrum and Roughton (5) has been used with some changes. The boat consisted of a 25 cc. Erlenmeyer flask with a wedge melted across the bottom. 1 cc.

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of 0.2 M sodium bicarbonate in 0.02 N KOH was placed in one compartment. 0.1 cc. of the enzyme solution and 1 cc. of phosphate buffer, pH 6.8, were placed in the other compartment of the boat. The determinations were run at 0°. The boat was connected to the water manometer, and after temperature equilibrium had been reached the reaction was started by rapid shaking

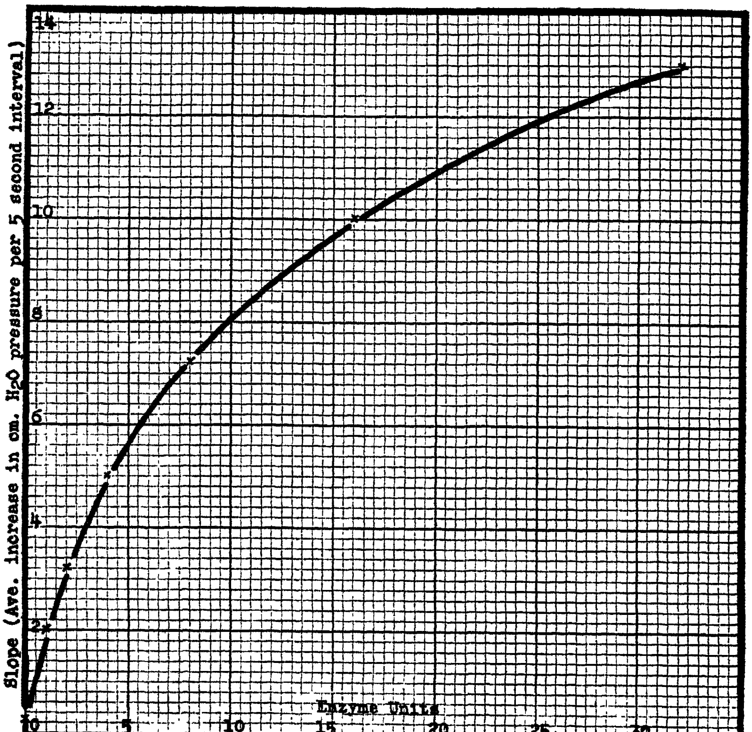

FIG. 1. Carbonic anhydrase standard curve at 0°. 1 unit is equivalent to 0.15 γ of purest enzyme preparation.

with an electric motor. The rate of this shaking was standardized at four back and forth swings through a 90° angle per second. Readings on the manometer were taken at 5 second intervals. The activity of the enzyme was expressed in terms of arbitrary units based on the maximum slopes of the CO_2 output curves. The standard curve, in terms of these arbitrary units, is shown in Fig. 1.

Method for Determination of Zinc—A dithizone colorimetric method has been used for the determination of zinc. A sample containing between 1 and 5 γ of zinc is dry-ashed, taken up in 1 cc. of 5 N HCl, and washed into a small separatory funnel with 9 cc. of water. This is shaken with 2 cc. portions of a purified dithizone solution (2.5 mg. per cent in CCl_4). A quantitative separation of the copper from zinc is thus effected. The CCl_4 phase containing the Cu, and other metals such as Ag and Hg if present, is discarded. The water phase in the separatory funnel is brought to pH 3.5 with 1.5 cc. of 5 N ammonium acetate. Violent shaking with 2 cc. portions of the dithizone solution now results in the complete extraction of zinc ions. The CCl_4 phases containing the red zinc dithizonate are run into a 25 cc. volumetric

TABLE I

Standards for Zinc Determination

	Galvanometer reading	L (density)	Ratio, $\frac{\gamma \text{ Zn}}{L}$
Blank	Set at 100	0	
1 γ zinc	78^3	0.104	9.61
2 " "	62^2	0.204	9.80
3 " "	50^0	0.301	9.96
5 " "	32^2	0.488	10.20
1 " " + 10 γ Pb + 10 γ Cu	80^1	0.097	10.40

flask. This has now effected a complete separation of the zinc from lead ions, the latter being untouched by dithizone at this pH. After appropriate washings, the excess dithizone in the volumetric flask is removed with dilute ammonia (0.1 per cent of a concentrated solution, distilled). The red zinc dithizonate is brought to volume with pure CCl_4 and read on the Evelyn colorimeter, Filter 520. A blank and standard are run with every determination. The results on pure zinc solution carried through the above procedure are shown in Table I.

Purification of Carbonic Anhydrase—Defibrinated beef blood obtained from a slaughter-house has been used as the source material. The cells were centrifuged down, thoroughly washed with 0.9 per cent saline, and lysed with an equal volume of 35 per cent alcohol. The hemoglobin was denatured by shaking with a 0.2 volume of distilled chloroform for 10 minutes, allowing to stand overnight

in the cold, and filtering on a Buchner funnel. The resulting solution had a clear, light amber color.

A preliminary acetone fractionation on this material was carried out. All operations were conducted in a cold room. The acetone and all other volatile reagents used in this work were redistilled from glass. In the first fractionation the crude chloroform preparation was treated with 1, 1.5, 2, 2.5, and 3 volumes of chilled acetone, in series. Fraction II (1.5 volumes of acetone) was found to have the highest specific activity (shown in Table II). A second acetone fractionation of Fraction II gave a somewhat increased specific activity (Fraction II-B of Table II). The zinc content of this second fraction was 0.171 per cent of the dry matter.

TABLE II

Purification of Carbonic Anhydrase by Acetone Fractionation

	Zn in dry matter	Specific activity (dry basis)	Yield of enzyme	Ratio, $\frac{\text{per cent Zn}}{\text{specific activity}}$
	per cent	*units per mg.*	*per cent*	
Lysed red blood cells	0.005	63	100	78×10^{-6}
$CHCl_3$ preparation	0.056	960	77	58
Fraction II (1.5 volumes acetone fraction)	0.144	2700	49	55
Fraction II-B (fractionation of Fraction II)	0.171	3330	28	51

A more intensive purification was now carried out, starting with the crude chloroform preparation of another batch of beef blood. It was found by trial that 10 cc. of a saturated lead subacetate solution per liter of preparation resulted in a voluminous precipitate but no loss of carbonic anhydrase activity. More than this amount of lead subacetate caused the marked precipitation of enzyme. Since Keilin and Mann (6) had used this proportion of the subacetate for the complete precipitation of the compound hemocuprein, this step should effect the separation of carbonic anhydrase from this copper-containing protein. After a 2 day dialysis in the cold, the liter of lead subacetate filtrate was treated several times with 25 cc. portions of an aluminum hydroxide cream (20 mg. per cc.). This particular batch of alumina cream

effected some purification by removal of impurities. 2 volumes of chilled acetone were added and the precipitate taken up in 200 cc. of water. The zinc content was now 0.271 per cent (Table III). Absolute alcohol was added to make a 65 per cent alcohol solution; the mixture was held at 45° for 15 minutes and centrifuged after rapid chilling. The precipitate was discarded. This step gave a slightly greater specific activity and raised the zinc content to 0.318 per cent on a dry basis. The enzyme was too unstable in this extreme dilution to attempt further purification. However, the report of Keilin and Mann (1) has been confirmed in all essentials. The concentration of the enzyme holds a con-

Table III

Purification of Carbonic Anhydrase

	Yield of enzyme	Dry matter	Zn content	Zn in dry matter	Activity		Ratio, per cent Zn / specific activity
	per cent	*mg. per cc.*	*γ per cc.*	*per cent*	*units per cc.*	*units per mg.**	
Lysed red blood cells.	100	121.5	2.34	0.002	7100	58	34×10^{-6}
$CHCl_3$ preparation..	55	2.7	2.06	0.077	4700	1740	45
Lead subacetate filtrate...........	49	1.8	1.77	0.098	4500	2500	40
$Al(OH)_3$ filtrate.....	32	0.84	1.40	0.167	2610	3100	45
Acetone fractionation..............	8	0.36	0.98	0.271	2030	5650	48
Alcohol (65 %) filtrate............	3	0.09	0.29	0.318	610	6700	47

* Dry basis.

stant relation to the concentration of zinc through every step of the purification, within experimental error.

The purified material was water-clear and colorless. It contained 15.2 per cent nitrogen, 0.006 per cent iron, and 0.03 per cent copper.

Since a lead salt was used in the purification, a special effort was made to establish the fact that none of this lead was entering into the zinc estimation. This was done by shaking the red zinc-dithizone complex (in CCl_4) with 10 cc. of 0.1 N NaCN. The color was completely removed. Under the same conditions it was found that a lead dithizonate was completely stable.

It should be emphasized that neither in this work nor in the original work of Keilin and Mann is there any indication that the maximum zinc concentration in carbonic anhydrase has been reached. The following may have some bearing on this point. An enzyme sample purified by acetone precipitation and containing 0.271 per cent of zinc was made 0.6 saturated with ammonium sulfate. The slight precipitate was filtered off and discarded after it showed negative activity. The filtrate, after thorough dialysis, had a zinc content, on the dry basis, of 0.51 per cent. However, the ratio of the specific activity to the zinc content was very low, and the specific activity itself (units per mg. of dry matter) was still about the same as before the ammonium sulfate treatment. Because of this it was concluded that no purification had been effected and the high zinc content was due to the introduction of extraneous zinc as an impurity in the ammonium sulfate. However, there is an alternative explanation. It is possible that traces of the ammonium ions brought about a 50 per cent inhibition, or that 50 per cent of the activity was destroyed during the long dialysis period, in which case, the 0.51 per cent zinc preparation would represent a true purification.

The dialysis of relatively concentrated solutions of the enzyme at pH levels of from 6 to 9 does not remove any of the activity or any of the zinc. Both zinc and activity are lost by dialysis below pH 5. The addition of inorganic zinc salts to this dialyzed material is unable to restore any part of the lost activity.

Effect of Inhibitors—Dithizone, which has an avid affinity for extremely small traces of ionic zinc in neutral or slightly alkaline solutions, is unable to produce any significant inhibition of carbonic anhydrase. However, the limited solubility of this dye in water is an important factor in this conclusion. A saturated solution (35 γ or 0.05 mM) does cause a 25 per cent decrease in activity, but smaller amounts have no effect.

Inorganic zinc in relatively large amounts (100 γ or a final concentration of 0.75 mM) produced a 50 to 60 per cent inhibition. 1 to 30 γ of zinc had no effect on the enzyme. However, a preformed zinc dithizonate (in 0.02 N KOH) equivalent to 10 γ of dithizone and 1.8 γ of zinc was able to inactivate completely the enzyme if added directly to the enzyme in the reaction flask. If added to the opposite side in the reaction flask, the inhibitory

effect was very much less (20 to 30 per cent). This property is not specific for the zinc dithizonate, for both lead and cadmium dithizonates acted the same. It is possible that this action is due to adsorption of the enzyme on the colloidal metal dithizonate particles. However, the activity could not be regenerated by bringing to pH 5 and extracting the free dithizone with CCl_4.

Davenport (7) has reported that the thiocyanate ion exerts a strong inhibition on carbonic anhydrase. This has been confirmed (Table IV). He suggests that this action was due to complex formation between one valence of the zinc of the enzyme and the thiocyanate. That this does not appear to be the case is suggested

TABLE IV

Inhibition of Carbonic Anhydrase by Thiocyanate and Zinc Ions

Addition	Activity	Per cent inhibition
	units per cc.	
None	649	0
KCNS, 10 mM	44	93
" 5 "	110	83
" 2 "	242	63
$ZnSO_4$, 1 "	240	63
KCNS (2 mM) + Zn (1 mM)	121	81

from results shown in Table IV. The thiocyanate inhibition could not be removed by a previous mixing of the thiocyanate with equivalent amounts of zinc. Both inhibitors appear to have independent action and do not neutralize each other.

Carbonic Anhydrase Content of Blood of Zinc-Deficient Rats—A zinc deficiency was produced in rats as described by Hove, Elvehjem, and Hart (2). The blood samples for carbonic anhydrase determinations were obtained by drawing 0.02 cc. of whole blood into a standard 5 cc. hemoglobin pipette and diluting to the mark with distilled water. Samples for hemoglobin determinations were taken at the same time. The determinations were made within an hour of the bleeding. The results of these determinations and of analysis of the red blood cells for zinc are shown in

Table V. There appears to be no very significant decrease in the carbonic anhydrase to hemoglobin ratio in the the zinc-deficient rats. Correspondingly, there is but a very slight drop in zinc concentration in the red blood cells. The zinc contents of the plasmas are not shown, since they have no direct bearing on the other determinations. These values varied between 2.5 and 4.0 γ of zinc per cc. of plasma, depending in part on the condition of the rat.

TABLE V

Carbonic Anhydrase, Hemoglobin, and Zinc Content of Bloods of Zinc-Deficient and Normal Control Rats

	10 Zn-low males		5 males, Zn added	
	Range	Average	Range	Average
Hemoglobin, %	10.7–14.6	12.76	12.0–15.8	13.50
Carbonic anhydrase, *units per c.mm.*	4.5– 6.5	4.80	5.0– 6.7	5.60
Carbonic anhydrase, *units per gm. Hb* $\times 10^4$	3.1– 4.9	3.81	3.5– 4.6	4.18
Zn in red blood cells, γ *per cc.*	12.9–14.4	13.4	13.0–15.7	14.1
	9 Zn-low females		5 females, Zn added	
Hemoglobin, %	10.0–14.2	12.08	13.5–14.3	13.78
Carbonic anhydrase, *units per c.mm.*	2.5– 5.0	3.90	4.7– 6.5	5.84
Carbonic anhydrase, *units per gm. Hb* $\times 10^4$	2.4– 4.0	3.27	3.5– 4.7	4.08
Zn in red blood cells, γ *per cc.*	10.7–15.5	13.4	15.0–16.5	15.7

The average gain per day during the week prior to these determinations varied from 0.2 to 1.3 gm. for the zinc-deficient group, and 2.5 to 4.6 gm. for the controls with zinc added.

The average daily gain in weight during the week previous to sampling was 0.2 to 1.3 gm. for the zinc-deficient animals, and 2.5 to 4.6 gm. per day for the controls with zinc added.

DISCUSSION

The observation of Keilin and Mann that carbonic anhydrase contains at least 0.3 per cent of zinc and that all of the zinc in the red blood cells is present in this enzyme has been confirmed. However, there is yet no clue as to the nature of the linkage binding

zinc to the rest of the enzyme molecule, nor to a possible prosthetic group.

According to Schorn (8) a protein solution such as "ash-free" egg albumin forms definite compounds with various metallic salts, in which 1 gm. equivalent of the metal is bound by 5200 gm. of the albumin. The metal cannot be removed by electrodialysis. On this basis the zinc-albumin compound would contain 0.63 per cent of zinc. It will be interesting to see if the carbonic anhydrase cannot be purified to the extent that it contains a higher amount of zinc than 0.3 per cent.

Carbonic anhydrase determinations were carried out on rats which were quite definitely suffering from a zinc deficiency; yet no very significant decrease was noted. There did appear to be a slight drop in hemoglobin in these rats. Since an anemia would be expected if the hematopoietic organs were unable to synthesize any single component of the red blood cells, whether hemoglobin or carbonic anhydrase, this fall in hemoglobin level in the zinc deficiency is not particularly strange. If a zinc deficiency could be produced which was so severe that the animals actually lost weight and died, it is possible that a decrease in the carbonic anhydrase content of the red blood cells would be found. In some of the rats described in a previous paper (2) a much more severe deficiency occurred, as judged by the growth records. By contrast, these rats did show a decreased zinc content of whole blood. In the two rats of this group which died, an extremely rapid, gasping type of respiration was evident for a week prior to death. There was some frothing and foaming at the mouth also. At autopsy the lungs showed no congestion, however.

Since carbonic anhydrase is primarily localized in the red blood cells, zinc must have other functions in other tissues. Davenport (7) has reported the occurrence of carbonic anhydrase in the parietal cells of the stomach mucosa, and van Goor (9) has reported its presence in the pancreas. Naturally, extreme care must be taken to obtain preparations of these organs which are absolutely blood-free. It is interesting to note that Holmberg (10) has reported that a partially purified uricase contained 0.13 per cent zinc.

In an interesting paper, Kiese and Hastings (11) have demonstrated that several oxidizing agents are strong carbonic anhy-

drase inhibitors. This inhibition could be reversed by reducing agents such as ascorbate and cysteine. The same authors (12) have shown, also, that certain inorganic substances have a non-enzymatic catalytic effect on the hydration and dehydration of CO_2. Bromine and chlorine had the strongest action, while iodine was ineffective.

SUMMARY

1. The report that carbonic anhydrase contains at least 0.3 per cent of zinc and that all of the zinc in the red blood cells is bound into this enzyme has been confirmed.

2. The highly sensitive zinc reagent, dithizone, produced little significant inhibition of carbonic anhydrase. KCNS was markedly inhibitory. An equivalent amount of zinc ion was unable to neutralize this inhibitory property of thiocyanate. Zinc ions alone, in relatively large amounts, produced a 50 to 60 per cent inhibition.

3. Under the experimental conditions employed there is but little significant decrease in the carbonic anhydrase to hemoglobin ratio in zinc-deficient rats.

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THE FACTOR V (COENZYMES I AND II) CONTENT OF RAT TISSUES: EVIDENCE FOR SYNTHESIS OF NICOTINIC ACID BY THE RAT

By W. J. DANN and HENRY I. KOHN

(*From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina*)

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Specific deficiency diseases caused by lack of dietary nicotinic acid have been described in the dog, pig, monkey, and man. In the dog and the pig it has been shown that the coenzyme content of the liver and voluntary muscle is markedly decreased in the deficient animal (1–3) but the coenzyme content of the blood cells is not decreased. An early report (4) suggested that the coenzyme content of the blood was lowered in deficient humans (pellagrins) but later work shows this to be incorrect (5, 6); therefore in three of the species the blood coenzyme level does not change significantly in the deficient state, but the level in certain other tissues has been shown to fall in the only two species examined.

No specific nicotinic acid deficiency disease has yet been reported in the rat, but on the basis of the results with other species it is to be expected that if such disease occurred it would be accompanied by large decreases in the coenzyme content of the liver and voluntary muscle, but little change in the blood level. We have sought evidence for the existence of such a disease by examining the coenzyme content of certain tissues of the rat on complete diets and on diets low in nicotinic acid. In addition we have obtained figures on nicotinic acid balance in an attempt to find whether the rat is dependent on dietary nicotinic acid.

Methods

The rats were taken from a breeding colony of the Vanderbilt strain (7), maintained on a stock diet of Purina Chow, bread, lettuce, corn-meal, whole wheat, pig liver, dried milk, cod liver oil, and salt.

Two diets were used in the study. Diet 1 was the modified Goldberger diet used in previous blacktongue studies (1), fed *ad libitum*, and supplemented daily by 20 γ each of thiamine chloride, riboflavin, and pyridoxin for each rat. This diet induces blacktongue (nicotinic acid deficiency) in dogs, but three of its ingredients contain small quantities of nicotinic acid. Acid hydrolysis of these materials, decolorization of the digests by the method of Melnick and Field (8), and estimation of the nicotinic acid by a König reaction with metol showed the nicotinic acid contents in micrograms per gm. to be for corn-meal 7.8, cow-peas 11.6, washed casein 5.7. The diet contained 13 per cent corn-meal, 1.27 per cent cow-peas, and 1.75 per cent washed casein; the water content was 80 per cent. The complete diet therefore contained 1.3 γ of nicotinic acid per gm.; reckoned on a dry weight basis, 6.5 γ pèr gm. On this diet weanling rats grew slowly, reaching a weight of 200 to 300 gm. in 6 to 9 months.

Diet 2 was designed to have an even lower nicotinic acid content than Diet 1. It contained Labco vitamin-free casein 20 per cent, sucrose 75 per cent, salts[1] 5 per cent, and was supplemented for each rat daily with 20 γ each of thiamine chloride, riboflavin, and pyridoxin[2] and with 2 drops of a yeast extract rich in pantothenic acid. It was also supplemented twice weekly with 2 drops of cod liver oil. The yeast preparation was a 55 per cent alcohol extract,[3] treated with phosphotungstic acid to remove nicotinic acid. This casein contained 3.6 γ of nicotinic acid per gm., and the complete basal diet 0.7 γ per gm. The daily dose of yeast extract for each rat contained 0.8 γ of nicotinic acid.

Rats were sacrificed as required for estimation of the tissue coenzymes. These were determined by the factor V method (9) which is sensitive to coenzymes I and II and possibly to closely related unknown substances. The analyses are expressed in diphosphopyridine nucleotide equivalents; *i.e.*, micrograms of diphosphopyridine nucleotide (coenzyme I) equivalent in activity to 1 gm. of wet tissue.

[1] The salt mixture was that described by Drummond and Watson, *Analyst*, 47, 235 (1922).

[2] These three vitamins were kindly furnished by Merck and Company, Inc.

[3] Kindly furnished by Mr. G. F. Siemers of Anheuser-Busch, Inc.

Results

Growth Rate on Diet 1 and Diet 2—The rats taken for experiment were 21 to 24 days old and most were between 35 and 50 gm. in weight. On Diet 1 with the supplements described above these rats grew slowly but steadily over long periods, and most of them remained active and healthy. Some died of intercurrent infections; a larger number than would have been expected if the stock diet had been used. In the longest trial made, lasting 9 months, a number of the rats grew to 300 gm. on Diet 1. The greatest growth rate observed for any rat was 2.5 gm. daily, maintained for 2 weeks; a rate of 2 gm. daily was commonly maintained for 6 to 10 weeks. The food consumption was measured for some groups and the mean value was 22 gm. daily during the first 6 weeks on the diet. The maximum food consumption observed in 1 day was 30 gm.

When Diet 1 was supplemented with 1 mg. or 2 mg. of nicotinic acid or nicotinamide daily, no significant difference in growth rate was observed in comparison with litter mate controls.

Rats given Diet 2 with the supplements described above grew 15 to 30 gm. in about 3 weeks and then remained almost constant in weight for long periods. Although stunted, they remained active and appeared to be in good condition. The mean food consumption was 7 gm. daily per rat. Tests controlled by untreated litter mates showed that the addition of 1 or 2 mg. of nicotinic acid daily did not cause increased growth.

Coenzyme Content of Tissues—Forty rats, in ten groups of four each, were sacrificed for estimation of the coenzyme content of liver, kidney, and thigh muscle. Each group was of the same age and dietary history, but different groups varied from 3 weeks to 6 months old. The 3 week-old rats were weanlings from the breeding colony; other groups had received one of three diets, (1) the stock diet of the breeding colony, (2) Diet 1 with 1 or 2 mg. of nicotinic acid daily, or (3) Diet 2 with 1 or 2 mg. of nicotinic acid daily. The figures obtained did not show any significant differences between the groups, even when the stunted rats kept on Diet 2 + nicotinic acid were compared with others of the same age on the stock diet, or when 3 week- and 6 month-old rats on the stock diet were compared. The results are therefore given

only as means, presented in Table I, and are regarded as normal values.

TABLE I

Coenzyme Content of Rat Tissues Determined by the Factor V Method

The coenzymes are expressed in micrograms of diphosphopyridine nucleotide equivalent to 1 gm. of wet tissue.

Tissue	No. of samples	Mean coenzyme content	Standard deviation
Liver	39	540	58
Kidney	38	519	71
Thigh muscle	36	524	60

TABLE II

Coenzyme Content of Tissues of Rats Maintained on Diets Deficient in Nicotinic Acid, and Effect of Adding Nicotinic Acid

The values are expressed in micrograms of diphosphopyridine nucleotide equivalent to 1 gm. of wet tissue.

		1	2	3	4	5	6	7*
Experiment No.		1	2	3	4	5	6	7*
Diet No.		1	1	1	2	2	2	2
Days on diet		56	59	84	21	24	31	60
Mean factor V, deficient diet only	Liver	404	446	586	449	434	480	
	Kidney	390	462	587	448	403	544	
	Thigh muscle	399	450	576	445	438†	464	
Mean factor V, deficient diet + nicotinic acid supplement	Liver	519	509	574	587	542	616	
	Kidney	434	480†	537	569	549	593	
	Thigh muscle	483†	493†	546	547	547	607†	
Ratio, deficient to supplemented	Liver	0.78	0.88	1.02	0.76	0.80	0.78	1.02
	Kidney	0.90	0.96	1.09	0.79	0.73	0.92	0.87
	Thigh muscle	0.83	0.91	1.05	0.81	0.80	0.76	0.98

Each figure is the mean of four, except when marked (†), when one of the four samples was lost in analysis.

* In Experiment 7 the tissues of the two groups of rats were compared with one another but not with the standard coenzyme preparation.

To find the effect of deprivation of dietary nicotinic acid on the coenzyme level, a number of similar experiments were set up. In each, a number of weanling rats were placed either on Diet 1 or

Diet 2, and divided into two matched groups of litter mates, of which one group received added nicotinic acid. After a chosen period, four rats on the diet not supplemented with nicotinic acid were killed, together with their four litter mates which had received added nicotinic acid, and the tissues were analyzed for coenzyme. The results of seven experiments are collected in Table II. Analysis of the data shows that there is a statistically significant decrease in the coenzyme content of the tissues of those rats receiving Diet 1 or Diet 2 without the nicotinic acid supplement. The decrease on the average is about 10 or 12 per cent for each of the three tissues.

DISCUSSION

In order to establish nicotinic acid as a vitamin for the rat it would be necessary to show that without dietary nicotinic acid the rat either fails to survive, fails to grow, or suffers a specific loss of function or deficiency disease. Such evidence has never been firmly established. Frost and Elvehjem (10) reported a growth-promoting action of nicotinamide when it was added to a deficient basal ration; but the growth did not begin until 2 weeks after the nicotinamide was added to the deficient diet. This suggests that the amide was not acting directly as a vitamin for the rat, because the other water-soluble factors (thiamine chloride, riboflavin, pyridoxin) all produce an immediate response when added to diets from which they have been excluded.

From the experiments reported above we draw the following conclusions. First, the rats can survive for long periods on Diet 1 and Diet 2. They grow slowly on Diet 1 and scarcely at all on Diet 2, but in neither case does the addition of nicotinic acid increase growth. Therefore a deficiency of nicotinic acid is not the factor limiting growth on these diets, and it appears that the nicotinic acid requirement of the rat must either be very small or non-existent. In the second place, the severe restriction of dietary nicotinic acid leads to a decrease in the nicotinamide-containing coenzymes of the tissues which is only slight. In the dog which suffers from blacktongue after deprivation of nicotinic acid, the level of coenzymes in the liver tissue may be down to less than 50 per cent of the normal value, and for voluntary muscle to 65 per cent. In the rat, the smaller decrease to about 90 per cent

of the usual value is in sharp contrast to this, and suggests that the rat is not suffering any serious change due to lack of dietary nicotinic acid. The synthesis of the coenzymes takes place to almost the same extent as when the rat receives an abundance of nicotinic acid.

The third point to be made is that a balance sheet can be drawn which indicates that the rat actually synthesizes nicotinic acid when it grows on Diet 1. A common rate of growth maintained for at least 3 weeks on Diet 1 by rats in our experiments was 2 gm. daily. The food intake was less than 30 gm. Assuming that 80 per cent of the weight increase occurred in the soft tissues (see Donaldson (11)), and that the mean level of coenzyme in the soft tissues was 450 γ of diphosphopyridine nucleotide equivalent per gm. (see Table II), then the rat was synthesizing $1.6 \times 450\ \gamma$ of diphosphopyridine nucleotide equivalents daily, containing about 144 γ of nicotinic acid. The dietary intake was less than $30 \times 1.3\ \gamma = 39\ \gamma$ of nicotinic acid daily. Thus the rat was ingesting less than 40 γ of nicotinic acid daily and laying down over 140 γ in the soft tissues in the form of coenzyme. In addition, some must have appeared in the hard tissues and it is probable also that urinary excretion of nicotinic acid occurred. There can be no doubt that the rat synthesized a good deal more nicotinic acid than it ingested.

The observation that in the growing rat on Diet 1 the tissue coenzyme level remained as high as in the rat failing to grow on Diet 2 indicates that the rat synthesizes enough nicotinic acid to allow the maintenance of this level of coenzyme (which may be regarded as a physiological norm) and perhaps no more. The 10 per cent higher level in the rat receiving abundant nicotinic acid may then be regarded as an effect of luxus consumption of nicotinic acid.

As the rat is able to synthesize nicotinic acid when fed on Diet 1, we conclude that this compound is not a vitamin for this species. This conclusion is opposed to that of von Euler *et al.* (12) who state "dass Nicotinsäureamid für den Rattenkörper eine unentbehrliche Verbindung von Vitamincharakter ist." This opinion is based on their finding that rats on regimens deficient in nicotinic acid had a coenzyme level only about half as great as that of rats receiving liberal amounts of nicotinic acid. Their figures were

expressed as an average level for the whole carcass, and the large decrease they report depends upon a smaller number of more variable figures than ours. We are unable to discuss these findings at greater length, since von Euler *et al.* have not published the details of their basal diet.

It is noteworthy that other workers (13), using the same technique and standard preparation of coenzyme, found mean values within 2 per cent of ours for rats of the Douredoure strain[4] more than 7 days old, when the diet consisted of Purina Chow only. During the 1st week of life they found the coenzyme content increased rapidly. On the other hand, the cozymase content of rat tissue has been reported (2) as about twice the value found by us. The analysis was by a fermentation test, and a different standard and mode of extraction were employed. The cause of this difference in absolute values is now under examination.

SUMMARY

On two diets low in nicotinic acid the rate of growth of rats was not increased by adding nicotinic acid. The coenzyme level (pyridine nucleotides) of the liver, kidney, and thigh muscle was decreased only about 10 per cent by the deprivation of nicotinic acid.

On one of the deficient diets the growing rats synthesized coenzymes at a rate utilizing more nicotinic acid than was ingested, showing that nicotinic acid itself was being synthesized by the rats.

The normal level of coenzymes in the tissues of rats receiving liberal amounts of nicotinic acid in the diet is 540 γ per gm. of liver, 519 γ per gm. of kidney, and 524 γ per gm. of thigh muscle.

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THE ESTIMATION OF PHOSPHATASE IN YEAST

By JAMES J. RAE AND EDNA V. EASTCOTT

(*From the Department of Chemistry, University of Toronto, Toronto, Canada*)

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For the determination of the phosphatase content of yeast many methods have been used (1–3). The usual method is to hydrolyze a glycerophosphate and measure the amount of inorganic phosphate liberated. King and Armstrong (4) have shown, however, that phenyl phosphate could be used as a substrate and the phenol determined. To be satisfactory the method chosen must give a measurable amount of hydrolysis in as short a time as possible and must not require too much yeast. The advantages in using only a small amount of yeast are that there may be only limited amounts available and it reduces the amounts of undesirable impurities frequently present.

The method finally chosen was to measure the phenol liberated from a mixture of 10 mg. of yeast and 10 cc. of 0.01 M disodium phenyl phosphate, buffered with acetate veronal at pH 4.1, after 20 minutes at 37°. A summary of the experiments on which these choices are based follows.

EXPERIMENTAL

The treatment of the yeast used in all the preliminary experiments (Yeast 40) involved washing by centrifugation, drying in air and *in vacuo* over calcium chloride, and finally grinding to pass a 40 mesh sieve.

The Folin and Ciocalteu (5) reagent used for the colorimetric analysis of phenol was found to be effective in stopping the hydrolysis.

Effect of Temperature on Rate of Hydrolysis—Yeast phosphatase is active between relatively wide limits of temperature (0–60°) and exhibits a maximum near 50°. For this reason 37° was chosen as the temperature for performing the hydrolyses because it gave

a high rate and was not too near the optimum temperature, above which the rate falls very rapidly.

Effect of pH on Rate of Hydrolysis—The optimum pH for the phosphatases in the yeasts used was in the neighborhood of 4.0. The buffers used were the acetate veronal buffers described by Michaelis (6). All pH values were determined electrometrically with a glass electrode.

Effect of Time on Rate of Hydrolysis—For the estimation of phosphatase it is highly desirable to measure the "initial rate" of hydrolysis. For this reason as short a time as possible consistent with convenient readings on the colorimeter should be used. 20 minutes were chosen as a suitable time for the hydrolysis.

Effect of Substrate Concentration on Rate of Hydrolysis—If the substrate concentration is too low, the substrate is almost completely hydrolyzed in a few minutes, and it would be difficult to distinguish between yeasts with high and low phosphatase contents. High concentrations of substrate are to be avoided, since the substrate is a fairly expensive chemical. For these reasons 0.01 M was the substrate concentration chosen. When sodium β-glycerophosphate is used as a substrate under the conditions mentioned above, the amount of hydrolysis is so small that some observers using this method have reported that no phosphatase was present in certain yeasts.

Effect of Yeast Concentration on Rate of Hydrolysis—As the yeast concentration increases, the rate of hydrolysis increases. At all concentrations the amount of phenol liberated is proportional to the amount of yeast used. However, since it is desirable to measure the phosphatase before an appreciable amount of the substrate has been hydrolyzed, the smaller the amount of yeast used, consistent with convenient readings on the colorimeter and accurate sampling, the better. For these reasons 10 mg. of yeast were chosen as a suitable amount for each phosphatase determination.

Effect of Products of Hydrolysis on Rate of Hydrolysis—Experiments have shown that the addition of phenol, even up to 50 times the amount liberated in the hydrolysis, has no appreciable inhibitory effect on the hydrolysis. Phosphate, on the other hand, above 0.002 M has an inhibitory effect. This is another argument in favor of using as short a time as possible for the phosphatase

tests. When excess phenol was added, King's (7) method for the determination of phosphate was used to measure the hydrolysis, and, when excess phosphate was added, Folin and Ciocalteu's method for the determination of phenol was used.

The procedure finally used is as follows: Into L-shaped rocker tubes, described by Eastcott (8), were put 7 cc. of acetate veronal buffer, pH 4.1, 2 cc. of disodium phenyl phosphate (substrate) 0.05 M, and 1 cc. of yeast suspension (10 mg. per cc.). After 20 minutes rocking, 5 cc. of the Folin and Ciocalteu reagent were added to each tube. The contents were mixed and filtered through a No. 42 Whatman filter paper. A 5 cc. aliquot of the filtrate was transferred to a 15 cc. volumetric flask, and 1.5 cc. of extra Folin and Ciocalteu reagent and 2.5 cc. of sodium carbonate solution (20 per cent anhydrous) were added. The solution was made to volume with distilled water and, after 20 minutes, the blue color produced was compared with a standard phenol solution containing 0.1 mg. of phenol per 10 cc. Control experiments were carried out with two tubes, one containing substrate and buffer, the other buffer and yeast. These controls showed either no blue color or only slight traces.

The phosphatase content of a variety of yeasts determined in this way is shown in Table I. The *phosphatase unit* is defined as the number of mg. of phenol liberated by 10 mg. of yeast at pH 4.1 in 20 minutes at 37° from a solution containing 0.01 M disodium phenyl phosphate.

Note on Yeasts Mentioned Above—Bakers' Yeasts 30, 40, and 50 were obtained from Standard Brands Ltd. Yeast 50 was supplied to us without the binder usually present in yeast cakes but was washed and dried before testing in the same way as was Yeast 40. Yeast 30 when received had already been dried, and so it was only ground before testing. It had been grown on a protein-rich medium.

The Toronto strain of *Saccharomyces cerevisiae* I, Hansen was the yeast used in those labeled Yeasts S1, S2, S3, and TJ. The differences obtained are due to the culture media on which the yeasts are grown. After growth the washing and drying treatment was the same for all.

Yeast S1 was grown on a standard culture medium containing 1 gm. of calcium carbonate, 100 gm. of dextrose, 4.2 gm. of potas-

sium dihydrogen phosphate, 8.4 gm. of ammonium nitrate, 0.7 gm. of calcium chloride (hydrated), and 2.1 gm. of hydrated magnesium sulfate per liter. Before making to volume inositol, Bios IIB, and calcium pantothenate were added. Yeast S2 had a different Bios IIB preparation added to the culture medium and was grown for 48 hours instead of the usual 24 hours. Yeast S3 had the same bios as did Yeast S2 but was only grown for 28 hours. Yeast TJ had the inositol, Bios IIB, and pantothenate replaced by 15 cc. of filtered tomato juice.

TABLE I

Determination of Phosphatase in Various Yeasts

The substrate concentration was 0.01 M disodium phenyl phosphate; time 20 minutes, pH 4.1, temperature 37°, yeast 10 mg. per cc.; the phosphatase unit is the number of mg. of phenol liberated.

Yeast No.	Phosphatase units
40, bakers'	0.609
30, "	0.480
50, "	0.631
S1, Toronto strain	0.930
S2, " "	0.183
S3, " "	0.923
TJ, " "	0.310
Brewers' yeast, bottom	0.354
" " top	0.218

The brewers' yeasts were obtained from Canada Bud Breweries, Ltd., and were washed and dried before testing.

SUMMARY

In this paper is presented a procedure for the determination of phosphatase in yeast based on King's application of the use of Folin and Ciocalteu's phenol determination to measure the phenol liberated from disodium phenyl phosphate. The method is preferable to those now in use because it is more rapid and less yeast is required for each determination. It is also preferable to the phosphate method, because yeasts almost always contain inorganic phosphate which appears in the controls, whereas none of the yeasts investigated contained phenol.

The effect of the composition of the medium in which the yeast grows on the phosphatase content of the yeast is being investigated.

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PROPERTIES OF THE BLOOD OF NEGROES AND WHITES IN RELATION TO CLIMATE AND SEASON

By D. B. DILL, J. W. WILSON, F. G. HALL, and SID ROBINSON

With the Technical Assistance of F. Consolazio

(From the Fatigue Laboratory, Harvard University, Boston, the Department of Zoology, Duke University, Durham, North Carolina, and the Department of Physiology, Indiana University, Bloomington)

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It is now 100 years since Mayer voyaged as physician on a Dutch vessel to Java and noticed that blood from a superficial vein was more red in that climate than in Germany. He mistakenly deduced that the production of heat and the process of oxidation must be less in torrid zones than in colder regions. Adolph (1) has pointed out some of the fallacies in Mayer's interpretations. Blood from an arm vein may vary in composition without corresponding variations in mixed venous blood; the blood supply to the skin serves the function of regulating temperature as well as of transporting gas. In extremely high temperatures it may become arterial in character, while in great cold it may be nearly deoxygenated.

Petersen (2) has suggested that venous blood reflects changes in the weather. It is his thesis that the pH, CO_2 content, and dependent properties of blood are so sensitive to temperature and barometric pressure that they respond in a predictable fashion to storm fronts. His work (2) contains a considerable body of data on the relation between the properties of blood and day-to-day variations in the weather. While we are prepared to accept the idea that there is some dependence of the properties of venous blood on weather, the degree of dependence of the properties of arterial blood on climate, weather, race, and the make-up of the individual is less clear. It is these latter questions with which we are here concerned.

The sites and seasons of our own observations are Boston through-

out the year, and Boulder City, Nevada, and Benoit, Mississippi, throughout the summer. Our subjects consist of male adult Whites and Negroes. We are indebted to Dr. Hastings and Dr. Shock for their having made available the dates corresponding to their published records on five men studied at Chicago throughout a period of 3 years (3). The maximum daily temperatures have been supplied by the United States Weather Bureau.

As intimated above, we have sought as a by-product of this study evidence of individual idiosyncrasies. It may be possible to characterize an individual as definitively by internal anatomical characteristics and by quantitative differences in function as by external anatomical features. While this possibility has not been explored in detail, there is much to be said in its support. Thompson, Corwin, and Aste-Salazar (4) have pointed out the dependence of the respiratory pattern, delineated on the pneumogram, on mental make-up and they have been able to associate certain types of pneumograms with certain mental illnesses. This implies that the functioning of the respiratory center is a distinctive physiological characteristic. Shock and Hastings (3) have shown that the cell volume and probably the $p\mathrm{CO}_2$ values for men and for women are significantly different. They also conclude that normal individuals differ significantly from one another in regard to the acid-base balance of their blood. The hemoglobin concentration in the blood of man reflects the balance between opposing forces, the formation and destruction of red cells, and hence this may be looked upon as giving a clue to the activity of certain tissues and organs. Similarly, the alkaline reserve (hereinafter referred to at T_{40} and defined as the CO_2-combining capacity of oxygenated blood at $p\mathrm{CO}_2 = 40$ mm. of Hg and at 37°) depends on the ingress and egress of acids and on certain processes of intermediary metabolism; the CO_2 content of arterial blood depends on alkaline reserve and respiratory regulation; the concentration of protein in serum depends on its rate of movement through capillaries, on shifts of water between extra- and intracellular spaces, and on the water balance of the body as a whole. We do not propose to consider many of these points in detail, but think it worth while to emphasize that idiosyncrasies of the subjects as well as racial differences may render more difficult an assay of the effects of season and climate.

The existence of physiological differences between Negroes and

Whites other than those dependent on pigmentation and external anatomical features is suggested by the superior records of Negroes in track and field athletics and by the commonly expressed opinion that they have greater resistance to high temperatures than have Whites. So far, however, no one has demonstrated unique physiological characteristics of the Negro that might be related to the capacity for energy transformation. During our experiments we found opportunities to test both the major premise and the deductions that logically follow.

Methods

With the exception of the Boulder City data, arterial CO_2 and O_2 content and capacity were determined in the usual manner and pH was calculated by the Henderson-Hasselbalch equation. Equilibration was carried out at 37°. At Boulder City the pH of arterial blood at 37° was determined directly by the glass electrode and T_{40} was calculated by use of data given by Peters and Van Slyke (5). Values for pH, determined at 37° on the glass electrode, and of arterial CO_2, determined on the Van Slyke apparatus, are applied to the line chart of Fig. 96 (p. 907 (5)). The slight degree of unsaturation of arterial blood is neglected and the factor f is read off the chart. This multiplied by whole blood CO_2 gives plasma CO_2. The next step consists in calculating the pCO_2 of arterial blood from the plasma CO_2, derived above, and the pH. Formula 5 of Table 57 (p. 881 (5)) may be used. Finally blood CO_2 at $pCO_2 = 40$ mm. of Hg may be estimated from the observed arterial CO_2 and from the calculated pCO_2 on the assumption that in normal blood the slope of the CO_2 dissociation curve is 0.24 mM of CO_2 per mm. of pCO_2, a relation implicit in Equation 26 (p. 912 (5)). In cases where the correction of blood CO_2 to a pCO_2 of 40 mm. exceeds 1 mM we employ the line chart of Henderson, Bock, Dill, and Edwards (6). With this chart, if the Hb and any pair of values of pCO_2 and CO_2 in oxygenated blood are known, it is possible to derive any other pair of values of the latter variables; *e.g.*, CO_2 when $pCO_2 = 40$ mm. of Hg which is T_{40}.

Results

Fig. 1 shows the values of pH in a group of 106 men in Boston (chiefly Harvard students) studied throughout the year and in two

groups studied in Benoit, Mississippi. Corresponding values for arterial CO_2 are found in Fig. 2. Despite the wide range of temperatures, there is no clear evidence of a dependence of these properties of the blood on the season. The trends of total CO_2, alkaline reserve, pCO_2, and pH with temperature were calculated

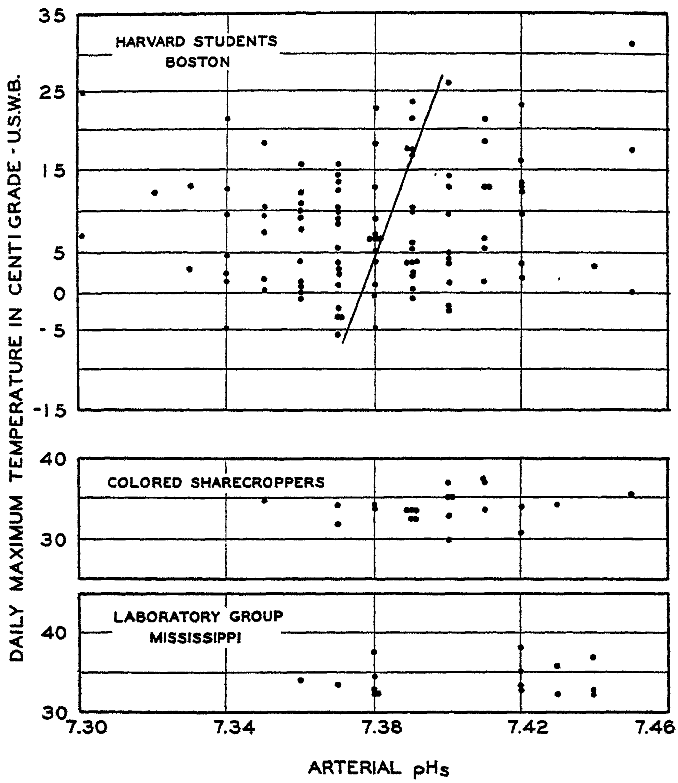

FIG. 1. Arterial pH in relation to daily maximum temperature. The line corresponds to the trend of the observations.

for the group of 106 students in Boston and for the 100 observations on five men made by Shock and Hastings in Chicago (3). As appears from Table I, there are slight positive trends with temperature of both arterial CO_2 and T_{40} in both places. The trends in pCO_2 and in pH were in opposite directions in both the

Boston and the Chicago data. If the two sets of data are put together, these opposite trends in pH and pCO_2 nearly counterbalance and the positive trends in CO_2 content and capacity are so slight that no convincing evidence is provided for any dependence of these functions on external temperature.

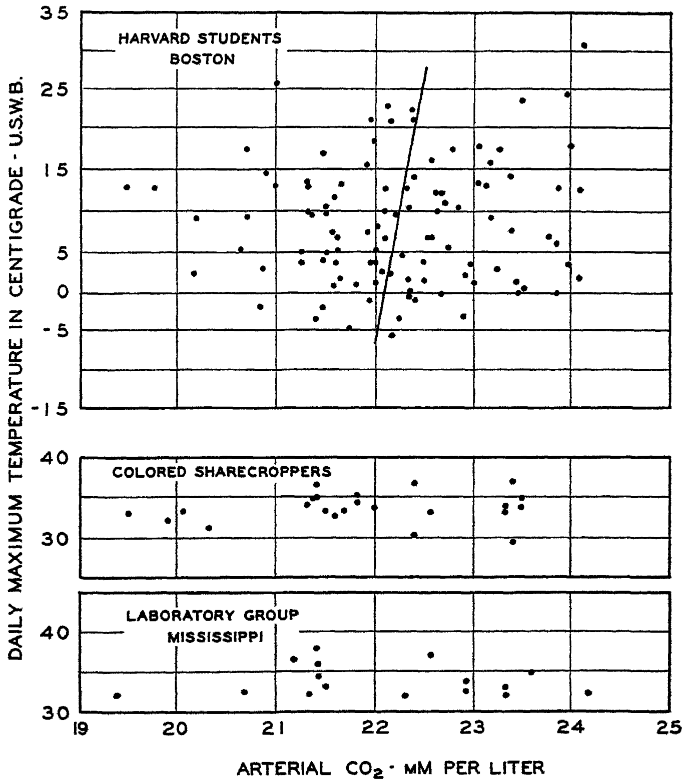

FIG. 2. Arterial CO_2 in relation to daily maximum temperature. The line corresponds to the trend of the observations.

Petersen's observations on pH of venous blood (2) were made by a colorimetric procedure. Venous blood is ordinarily more acid than arterial blood by about 0.03 pH unit and yet Petersen's pH values are more alkaline by about 0.05 than our observations on arterial blood. This implies a difference of about 0.08 between

pH as determined by Petersen, on the one hand, and by the Hastings colorimetric method, the equilibration procedure, or the electrometric method, with the glass electrode, on the other. Petersen's pH data on each of four men during 2 summer months in Chicago are shown in Fig. 3. There is a small but consistent trend in pH for each subject, amounting to about +0.0015 pH unit per 1°; for a temperature difference of +20° there is a pH increment of 0.03. This amounts to about the normal difference between arterial and venous blood. If arterial blood remains unaffected by temperature, it would be possible to account for

TABLE I

Trends in Composition of Arterial Blood with Temperature

The trends are calculated by the Pearson formula from the day's maximum temperature reported by the United States Weather Bureau.

Observers	Shock-Hastings	Dill *et al.*
Place	Chicago	Boston
No. of subjects	5	106
" " observations	100	106
Mean of maximum temperatures, °*C*	9	10
" total CO_2, *mM per l*	21.7	22.2
$\Delta CO_2/\Delta t$	+0.02	+0.01
Mean T_{40},* *mM per l*	21.3	21.5
$\Delta T_{40}/\Delta t$	+0.01	+0.02
Mean pCO_2, *mm. Hg*	41.2	43.4
$\Delta pCO_2/\Delta t$	+0.09	−0.04
Mean pH_s	7.395	7.381
$\Delta pH_s/\Delta t$	−0.0005	+0.0008

* T_{40} or alkaline reserve is defined as the CO_2 content of oxygenated blood equilibrated at 37° at pCO_2 = 40 mm. of Hg.

this great a change in venous pH by assuming that skin circulation increases so much in high temperatures that venous blood draining the skin capillaries remains virtually arterialized.

We have made similar measurements on members of our party in Boston and in summer expeditions to the desert and to the humid heat of Mississippi. The values of pH and of arterial CO_2 in desert heat are shown in relation to daily maximum temperature in Fig. 4. Two of the men, Adolph and Dill, are in a separate class in so far as arterial CO_2 content is concerned. Eleven observations on Adolph and Dill range from 21.9 to 25.0 mM per liter,

while twenty-two observations on the other five men range from 19.4 to 22.0 mM. A similar difference was observed in winter, and hence we have here individual idiosyncrasies that persist despite changes in climate and season. Aside from this feature,

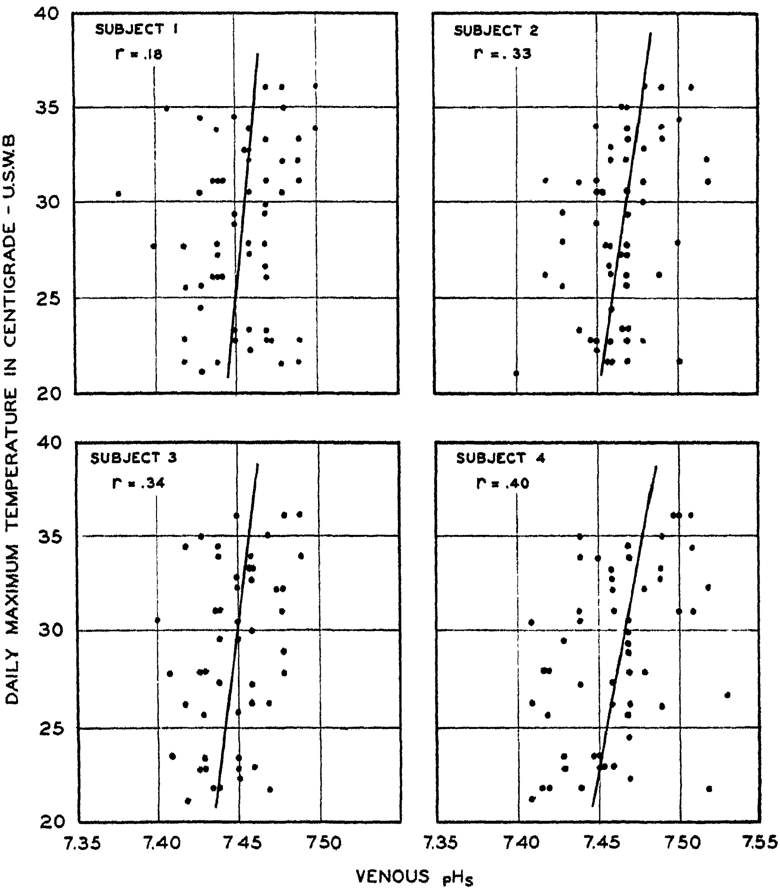

FIG. 3. Venous pH_s in four subjects studied by Petersen (2) in Chicago. r is Pearson's coefficient of correlation.

the only other comment to be made on Fig. 3 is that there is no clear relation between the fluctuations in daily maximum temperature and these properties of blood.

No racial difference was found in respect to the mean values of

arterial CO_2 and pH. This is evident not only from Figs. 1 and 2 but also from Table II; the mean values for these two functions in two groups of Negroes lie within the limits set by groups of white subjects with one exception.

The foregoing observations give no indication that two selected properties of the blood vary with season, but they do not disprove the contention of Petersen that there are day-to-day fluctuations in these properties that depend on the passage of storm fronts. If there is such a dependence, variability in the properties of blood

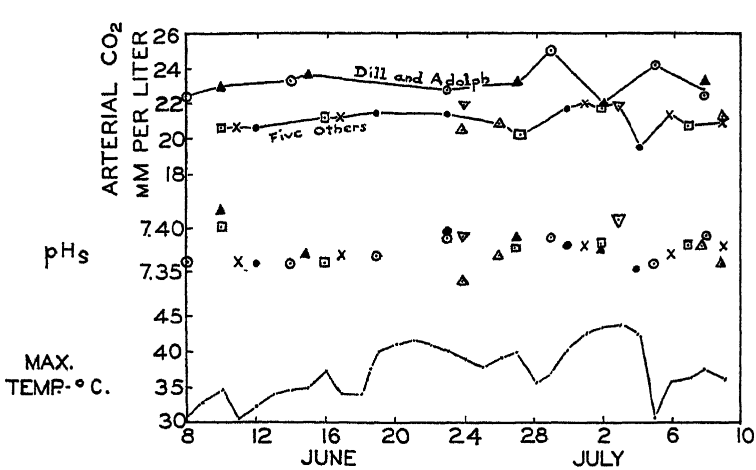

FIG. 4. Arterial CO_2 and pH during the summer of 1937 at Boulder City, a separate symbol being used for each of the seven men studied. No consistent differences were seen in pH but two men had a higher CO_2 content than the others. Neither function varied with the outdoor maximum temperature.

should be maximal in our Boston data and in those of Shock and Hastings (3) in Chicago. These measurements were made in all four seasons and in climates notable for instability. The least variability should be found in our measurements in Benoit where the summer weather is notable for its uniformity. During our stay at Benoit the barometric readings, uncorrected for altitude, averaged 749.2 mm. of Hg, with a standard deviation of 2.6. For a corresponding period in the summer in Boston the values were 763.3 ± 3.7 mm. and in the winter 764.1 ± 7.9. We have ac-

cordingly paid particular attention to the variability of pH and arterial CO_2 with respect to climate and season.

The possibility also exists that there are racial differences in variability even though the mean values are not significantly different. Negroes are reputed to have a different emotional

TABLE II

Arterial pH and CO_2 Content (in mM per Liter) in Relation to Climate and Race

Place	Subjects	No. of subjects	No. of measurements	Range of maximum temperature	pH		Arterial CO_2	
					Mean	S.D.	Mean	S.D.
				°C.				
Boston	Laboratory staff	10	10	0–15	7.386	0.018	22.12	0.84
Benoit	" "	10	16	32–38	7.403	0.026	22.09	1.22
Boston and Bloomington	Colored students	12	12	0–15	7.390	0.020	22.56	1.25
Benoit	Colored share croppers	23	23	29–37	7.398	0.021	21.89	1.17
Boston	Students and faculty	106	106	−5–31	7.381	0.030	22.22	1.00
Chicago	Shock and Hastings (3)*	5	100	−17–37	7.395	0.032	21.65	1.38
"	Shock and Hastings (7)*	39	104		7.404	0.027	22.30	1.20
"	Petersen (2)†	4	223	20–36	7.456	0.027	28.45	1.34

* The pH values of Shock and Hastings (3, 7) were determined colorimetrically at 38°. We have introduced a correction of +0.005 to bring them into line with our measurements made at 37°. We have employed only their first observation of each day whenever several were made on the same day.

† Venous blood. The method of Myers and Muntwyler (8) was used for pH determination. We have read off values from the curves given by Petersen ((2) Fig. 76).

make-up than Whites and it is true that emotional make-up can be reflected in the properties of blood obtained by puncturing an artery, or even a finger. While most subjects are little concerned, many show a respiratory response which may take the form of holding the breath in some or of overventilation in others.

We find that the arterial pH does not show consistent differences

TABLE III

Properties of Arterial Blood

Subjects	Place	HbO_2 capacity	HbO_2 content	T_{40}*	(Total CO_2)$_b$	pCO_2	(Protein)$_s$	(HCO_3)$_s$	(Cl)$_s$	(Lactate)$_s$	(Na)$_s$	(K)$_s$
		mM per l.	*per cent*	*mM per l.*	*mM per l.*	*mm. Hg*	*gm. per l.*	*m.eq. per l.*	*m.eq. per l.*	*m.eq. per l.*	*m.eq. per l.*	*m.eq. per l.*
Laboratory staff	Boston	8.92	95.1	21.4	22.1	42.8	66.0	25.2	105.6	1.6	139.7	4.6
" "	Benoit	8.68	96.1	21.7	22.1	41.1	63.1	25.2	106.0	2.1	140.0	5.4
Negro students	Boston and Bloomington	8.20	95.4	21.8	22.6	42.7	67.0	25.5	101.4	1.6	139.5	5.6
" share croppers†	Benoit	8.18	95.4	21.6	21.9	40.8	68.0	24.8	105.1	2.6	140.1	5.1
White " "	"	8.98	96.4	21.8	22.1	41.4	68.6	25.3	102.8	2.2	140.4	5.8

The subscripts *b* and *s* refer to whole blood and true serum, respectively.

* T_{40} or alkaline reserve is defined as the CO_2 content of oxygenated blood equilibrated at 37° at pCO_2 = 40 mm. of Hg.

† Includes two house servants.

in variability that can be related to climate or race (Table II). The smallest standard deviation was found in our laboratory staff in Boston; it was considerably greater in the same group in Mississippi. Negroes exhibited the same variability in Boston and Bloomington as in Benoit. While the greatest deviations occurred in the three large bodies of data collected in Boston and Chicago, we are not inclined to attach great significance to this, particularly since the smallest deviation in arterial CO_2 was found in the two Boston groups. The only indication in Table II that the races may differ lies in the small standard deviation in pH in the two groups of Negroes. However, their arterial CO_2 is not unusually constant.

Other properties of arterial blood are summarized in Table III. There are only a few points in Table III that deserve special mention. The hemoglobin in Negroes is about 8 per cent less than in the white subjects, including white share croppers. This we believe to be significant; the data will be presented in more detail elsewhere in connection with morphological studies of the blood. The serum protein in our party was unusually low in Mississippi, although it was within the usual limits in other groups there. It therefore appears that "thin" blood, while not uncommon in the South, is not necessarily a consequence of long residence there. It appears to be a racial characteristic of Negroes to have thin blood in the sense of low hemoglobin and it also seems that in the course of acclimatization Whites may undergo a temporary reduction in serum protein concentration. Finally, there are two groups in which serum chloride concentrations are below the usual range; these are the northern Negroes and the white share croppers. For this we have no explanation.

SUMMARY

The properties of arterial blood do not show a clear cut dependence on climate or season, nor is the standard deviation in pH and arterial CO_2, observed throughout the year in changeable climates, much greater than during the summer in the uniform humid heat of Mississippi. Blood from superficial veins appears to be more alkaline in hot weather; probably this is partially dependent on increased flow of blood through the skin. Negroes have less hemoglobin than Whites and their arterial pH may be

less variable than that of Whites. There is no other evidence for racial peculiarities in serum electrolytes.

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THE EFFECT OF SODIUM CHLORIDE ON THE GLUCOSE TOLERANCE OF THE DIABETIC RAT*

By JAMES M. ORTEN and HENRY B. DEVLIN†

(*From the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit*)

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During a study to determine the effect of certain inorganic substances on the glucose tolerance of the adult albino rat, it was observed that some animals of the strain employed (Connecticut Agricultural Experiment Station) showed a low tolerance to the intraperitoneally administered sugar. The glucose tolerance curves obtained on these rats were typically "diabetic" in nature. At this time a similar observation (1) was reported; 60 per cent of the Yale strain of rats 90 days of age or older showed a poor tolerance to glucose, whereas rats of the Wistar strain had a normal tolerance. Some animals, however, had a nearly normal tolerance to glucose at one test period whereas at another period they showed a low tolerance. The incidence of the low glucose tolerance was less in younger animals, all rats 50 days of age or less having normal tolerance curves. Some dysfunction of the anterior pituitary has been suggested (4) as a possible cause of the diabetic tendency in the Yale strain of rats.

In preliminary studies, the blood sugar values obtained on our rats with a low glucose tolerance showed an unusual trend. There was the expected sharp rise to a high value 30 minutes after the injection of glucose followed by a decrease after the 60 and 90 minute intervals. Then there was usually a secondary rise which

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† The data herein presented are taken from a dissertation submitted by Henry B. Devlin in partial fulfilment of the requirements for the degree of Master of Science in Wayne University, 1939.

frequently exceeded the first and which was sustained during the remainder of a 5 hour period of observation. During the secondary hyperglycemia, the animals frequently became cyanotic and exhibited tetanic contractions of various muscles. The seizures lasted for as long as 2 or 3 hours. These symptoms suggested that a disturbed electrolyte balance, possibly a lowered sodium to potassium ratio, might be involved. Considerable evidence supporting such an explanation is available. It has been shown (3), for example, that the intraperitoneal injection of solutions of glucose causes a migration of sodium and chloride ions into the injected fluid, thus producing a decrease in serum sodium and chloride. Other evidence showing a relationship between electrolyte balance and the metabolism of carbohydrate will be discussed later.

The present investigation was designed to study further the possible relation of sodium chloride to the utilization of glucose by rats showing either a hereditary diabetic tendency or a mild diabetes resulting from partial pancreatectomy.

EXPERIMENTAL

Adult male albino rats of the Connecticut Agricultural Experiment Station strain fed a stock colony ration were used. Pancreatectomized rats were prepared by the usual surgical procedure which produces a mildly diabetic animal not requiring insulin.

A standardized glucose tolerance test was performed on the rats after they had been fasted for 16 to 18 hours. Blood samples were taken from a tail vein 30, 60, 90, 120, 180, and 300 minutes, respectively, after the intraperitoneal administration of 0.35 gm. of anhydrous glucose (as an 8.75 per cent solution) per 100 gm. of body weight. Blood sugar was determined by the Somogyi micromodification of the Shaffer-Hartmann method (6) on a zinc sulfate-sodium hydroxide filtrate. Extreme care was taken to prevent any loss of sugar by glycolysis.

The tolerance to glucose alone was determined three times on every control and experimental animal. An interval of at least 10 days was allowed to elapse between tests. The entire procedure was then repeated on each animal but this time a glucose solution containing *sodium chloride* was injected. The glucose solution was made to contain 1.58 per cent sodium chloride, which

is isotonic with 8.75 per cent glucose. This concentration of sodium chloride was used in order to prevent any migration of sodium or chloride ions from the plasma into the injected fluid.

Results

It is evident from the averaged data given in Table I that all of the intact animals did not respond normally to intraperitoneally injected glucose. Approximately 30 per cent of the rats showed a

TABLE I

Blood Sugar Values of Intact and Pancreatectomized Rats Administered Glucose by Intraperitoneal Injection

350 mg. of C.P. glucose per 100 gm. of body weight were administered as an 8.75 per cent solution. The blood sugar values are given in mg. per cent.

Group No.	No. of rats	"True" blood sugar						
		Fasting	Min. after glucose administration					
			30	60	90	120	180	300
1. Intact rats, normal tolerance	25	82	207	201	184	159	156	127
		130	284	288	276	250	331	178
		47	132	116	99	98	90	72
2. Intact rats, low tolerance	11	75	268	243	231	224	257	295
		103	655	493	485	450	490	499
		55	169	184	164	140	164	182
3. Partially pancreatectomized rats	8	89	309	318	322	320	337	307
		100	381	447	476	474	404	525
		71	226	184	216	291	258	128

The figures for each group represent average, maximum, and minimum values, respectively.

low tolerance to glucose, as determined by the arbitrary criterion (1) of a blood sugar content of less than 180 mg. per cent at the end of a 5 hour period as the upper limit for a normal tolerance. The poor tolerance of these animals to glucose resembles that of the partially pancreatectomized animals, except for the fact that the latter did not show an early decrease and a subsequent rise in blood sugar values which characterize the usual response of intact rats with a low glucose tolerance. It should be added, however, that the response of some of the rats having a low

tolerance was not invariably the same. As has also been observed by Cole and Harned (1), alternations between normal and poor tolerances were found in a few animals. These rats were arbitrarily classified in the low tolerance group.

The fact that the volume of the injected solution and its hypertonicity were not responsible *per se* for the hyperglycemia and tetanic seizures in the rats having a low glucose tolerance was demonstrated by the intraperitoneal administration of volumes of isotonic solutions of sodium chloride (1.58 per cent) or of urea

TABLE II

Blood Sugar Values of Intact and Pancreatectomized Rats Administered Glucose with Sodium Chloride by Intraperitoneal Injection

The solution injected contained 8.75 per cent glucose and 1.58 per cent sodium chloride. The blood sugar values are given in mg. per cent.

Group No.	No. of rats	"True" blood sugar						
		Fasting	Min. after glucose administration					
			30	60	90	120	180	300
1. Intact rats, normal tolerance	7	81	217	214	162	150	128	99
		95	266	224	192	173	156	137
		73	241	204	145	136	116	85
2. Intact rats, low tolerance	6	74	276	248	220	194	154	119
		95	318	292	256	228	174	150
		64	204	216	168	176	120	81
3. Partially pancreatectomized rats	5	90	319	240	176	155	121	104
		156	429	419	368	344	238	162
		66	260	180	105	105	76	76

The figures for each group represent average, maximum, and minimum values, respectively.

(2.91 per cent) equal to that of the glucose solution used. No significant alteration in the blood sugar level occurred within 5 hours.

The results obtained on the animals given glucose with sodium chloride are given in Table II. The tolerances to glucose of the normal intact animals were but slightly lowered by the administration of sodium chloride. However, the intact rats with a low glucose tolerance and the pancreatectomized rats now showed a normal tolerance to glucose. Moreover, none of the animals which received the solution of sodium chloride and glucose mani-

fested the symptoms of muscular tetany seen in the same animals given glucose alone. An excessive water intake was observed in these animals, an observation previously recorded by Darrow and Yannet (3) for animals injected with a hypertonic solution of sodium chloride.

Another small group of animals, the data for which are not included in Table II, was injected with a solution of glucose containing 0.9 per cent sodium chloride. Again there was observed a normal tolerance to glucose.

DISCUSSION

The observation that sodium chloride improves the tolerance of the diabetic rat to intraperitoneally administered glucose is supported by the results of other types of experiments. For example, McQuarrie and coworkers (5) have found that in some human diabetics the ingestion of sodium chloride improves the utilization of carbohydrate. The improvement is manifested by a protein-sparing action, decreased glycosuria, lowered fasting blood sugar, increased respiratory quotient, and decreased ketosis. Furthermore, sodium chloride administration to rats increases the deposition of glycogen in the liver (2).

It also appears possible that the prevention of an altered electrolyte balance by the administered sodium chloride may explain its favorable effect on the glucose tolerance. The intraperitoneal injection of solutions of glucose alone is known to cause a migration of sodium and chloride ions into the injected fluid and to cause a decrease in the concentration of these ions in the serum (3). A decreased sodium to potassium ratio in the plasma could therefore occur. This alteration itself might conceivably produce a hyperglycemia and impair the utilization of the injected glucose, since it has been shown (7) that the injection of subtoxic amounts of potassium salts into rats produces a marked rise in blood sugar and a decrease in liver, muscle, and cardiac glycogen. Furthermore, the administration of potassium chloride causes an increased hyperglycemia in some diabetic patients (5). Thus, sodium and potassium ions appear to have antagonistic effects on carbohydrate metabolism.

In the present experiments, the favorable effect of sodium chloride might be explained therefore in either of two ways. It

appears possible that the secondary rise observed in the blood sugar values of the intact rats having a low glucose tolerance when they are injected with glucose alone might be a result of a decrease in the sodium to potassium ratio in the body fluids and tissues. The administration of sodium as the chloride could obviously prevent such a lowering of the ratio. On the other hand, the presence of sodium chloride in the solution of glucose may have exerted a direct favorable effect on the utilization of glucose, perhaps by way of an augmented rate of glycogenesis.

The possibility that the addition of sodium chloride to the injected glucose may have produced an increased diuresis and an increased renal excretion of glucose should also be considered. Preliminary qualitative experiments have indicated that this factor is of little importance in improving the glucose tolerance. Others (5) have even observed a *decreased* glycosuria in some human diabetics after the administration of sodium chloride. However, further quantitative studies on this question are now in progress.

An explanation of the reason why some intact rats show a normal tolerance to glucose without added sodium chloride whereas other intact rats and partially pancreatectomized animals show a normal tolerance only when sodium chloride is also injected is not possible at the present time. Obviously, further experimental work is needed to elucidate these questions.

SUMMARY

A study of the effect of sodium chloride on the glucose tolerance of intact rats with a low tolerance and of partially pancreatectomized diabetic rats has been made.

In contrast to the typically diabetic tolerance shown to glucose alone, the tolerances of both types of diabetic rats were normal when glucose was administered with sodium chloride.

The possible importance of the sodium chloride in increasing glycogenesis or in preventing a hyperglycemia due to a decreased sodium to potassium ratio or in increasing the renal excretion of glucose is discussed.

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RESYNTHESIS OF MUSCLE GLYCOGEN AFTER EXERCISE

By EUNICE V. FLOCK and JESSE L. BOLLMAN

(*From the Division of Experimental Medicine, The Mayo Foundation, Rochester, Minnesota*)

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Studies in the literature on resynthesis of glycogen after exercise show that it occurs even in isolated muscles, although in these the conditions are obviously far from optimal. Fletcher and Hopkins (1) (1907) found a 50 mg. decrease in lactic acid in isolated frog legs in a 2 hour rest period after work. Even according to the subsequently developed Hill-Meyerhof theory, this could account for only a small increase in glycogen. Meyerhof (2) (1920) found in isolated frog legs which had been stimulated to work for 15 to 30 minutes and then were allowed to rest in oxygen for 21 to 23 hours an average resynthesis of 123 mg. of glycogen.

The resynthesis of glycogen in mammalian muscles after work *in vivo* has also been shown to be rather a slow process. After contractions affecting practically all the musculature of the intact rat Long and Grant (3) (1930) found that resynthesis of glycogen was slow and took much more time than the removal of lactic acid. An increase of 55 mg. of glycogen occurred in 2 hours but complete recovery took 12 hours. Cori and Cori (4) (1933) found a more rapid resynthesis after work (a 15 second tetanus) which did not greatly deplete the glycogen stores; namely, an average of about 5 mg. per 100 gm. per minute over a 20 minute rest period. No increase during 30 minutes of rest after a 30 second tetanus in rabbits was found by Sacks and Sacks (5) (1935). Flock, Ingle, and Bollman (6) found that resynthesis of glycogen during rest in rat muscles which had been stimulated to contract three times a second for varying time intervals was a slow process and did not occur during the 20 minute period after the starting of work when the lactic acid content of the muscles was returning

to normal levels. No increase in concentration of glycogen was found during continuous work. We have now made further studies on the rates of breakdown and resynthesis of glycogen in rat muscles during and after work.

Procedure

Male rats weighing from about 180 to 200 gm. were anesthetized with sodium pentobarbital (nembutal) and the skin was loosened from the hind legs. The Achilles tendon of the left leg was loaded with a weight of 100 gm. and the muscle stimulated directly three times a second, each time with one complete cycle of 60 cycle current. The apparatus[1] used for stimulation consisted essentially of a disk which rotates three times a second, with a movable brush adjusted to make contact in a sector one-twentieth of the circle, thus sending a complete cycle of 60 cycle current through the muscle three times each second with a voltage of 50 through a resistance of 20,000 ohms. After varying intervals of work and rest this leg was quickly removed and frozen in a mixture of carbon dioxide ice and alcohol. The opposite control leg was then removed immediately in the same manner. Determinations of glycogen were made by a modified Pflüger method, of lactic acid by the method of Friedemann and Graeser (7), and of blood sugar by the Shaffer-Hartmann-Somogyi method (8).

Results

The average content of glycogen in the muscles of 94 control resting legs expressed as mg. of glucose per 100 gm. of muscle was 470 mg. Analyses of stimulated muscles showed that a rapid drop in glycogen occurred with 1 minute of work, an average amount of 265 mg. disappearing during this time (Fig. 1). The breakdown of glycogen proceeded for 2 additional minutes at the diminished rate of about 36 mg. per minute. Further breakdown occurred at a greatly reduced rate, if at all, but the glycogen did not disappear completely.

When the muscles were allowed to rest after a 3 minute period of work, there occurred a gradual increase in glycogen with considerable variation in degree. When the glycogen values were

[1] We are indebted to Dr. E. J. Baldes for the construction of the apparatus.

compared with the average content of glycogen in the muscles of forty-two other rats which worked 3 minutes only, it appeared that there is little if any increase in 20 minutes, some in 30, an

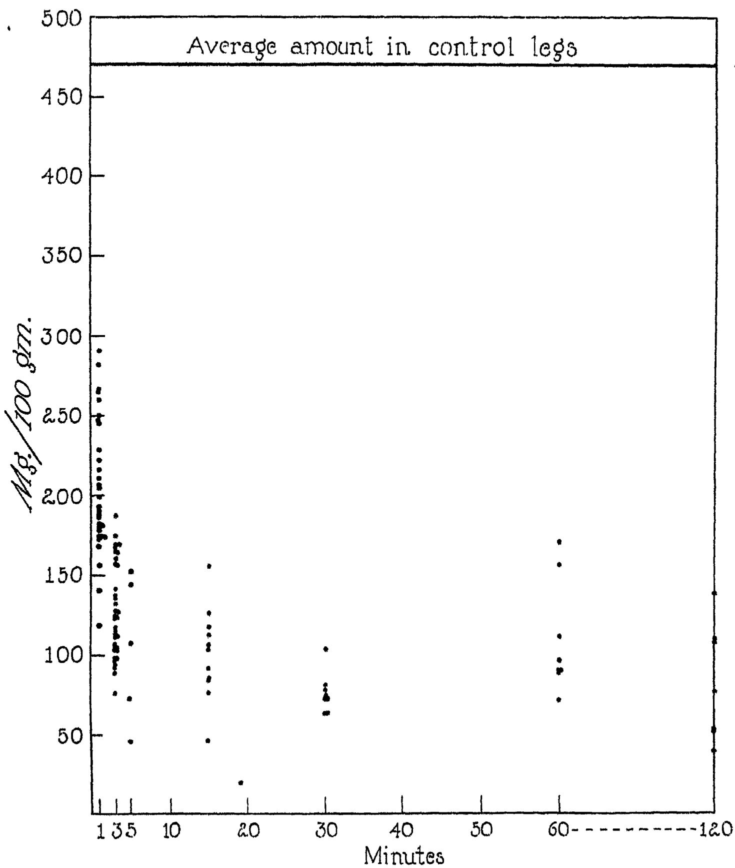

FIG. 1. Muscle glycogen during continuous work. The dots represent the content of glycogen in the working legs after varying intervals of continuous work. The average amount in 94 control resting legs was 470 mg.; standard deviation 71 mg.

average of 170 mg. in 1 hour, and an average of 200 mg. in 2 hours, or less than 2 mg. per 100 gm. per minute during the 2 hour period (Fig. 2). A similar study was made on muscles which

worked for 1 minute only and were then allowed to rest. In these, resynthesis started with the glycogen at a higher level,

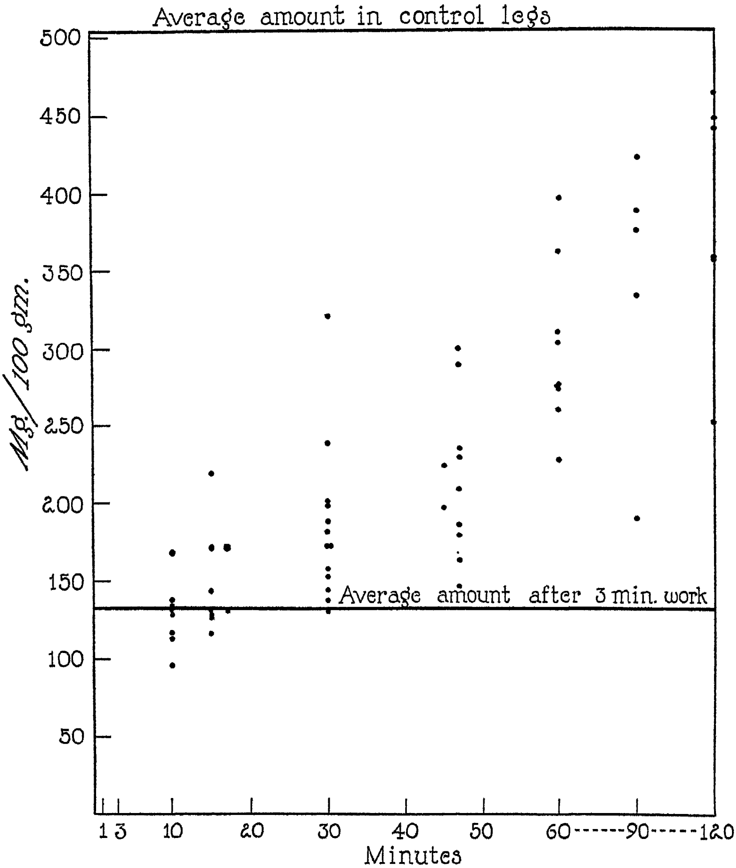

FIG. 2. Muscle glycogen during work and rest. The dots represent the content of glycogen at varying intervals after the beginning of work. During the first 3 minutes the rats worked, and then rested. The average amount in thirty-six control resting legs was 505 mg., standard deviation 96; the average amount in forty-two legs which worked for 3 minutes was 133 mg., standard deviation 34.

that is on the average 205 mg. in contrast to 133 mg. after 3 minutes work, and proceeded at a somewhat faster rate during the 1st hour but again averaged about 2 mg. per 100 gm. per

minute over a 2 hour period. A limited number of rats were allowed to rest for 2½ to 6 hours after 1 minute of work and in these practically complete resynthesis was found in all cases.

This slow, spontaneous resynthesis of muscle glycogen which proceeds after the cessation of work is not greatly modified even with great variations in the carbohydrate content of the circulating blood. No effect was noted when lactic acid, largely as the sodium salt, was given intraperitoneally in amounts of 100 mg. per 100 gm. before the stimulation and at 20 minute intervals

TABLE I

Effect of Epinephrine during Period of Resynthesis*

Rat No.	Time of rest after 3 min. work	Glycogen content		Blood sugar
		Worked leg	Control leg	
	min.	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 ml.*
1	27	197	437	142
2	27	129	509	172
3	27	122	450	125
4	57	244	323	185
5	57	188	315	202
6	87	323	267	255
7	87	215	232	245
8	87	114	279	77
9	117	245	166	265
10	117	509	289	230
11	117	463	325	198
12	117	183	149	148

* Administered to rats in amounts of 0.1 cc. of 1:10,000 solution of epinephrine per 100 gm. before stimulation and at 20 minute intervals thereafter, except for Rats 8 and 12 which received 0.5 cc. quantities. The rats had been fasted 18 hours.

thereafter. No effect was noted when the blood sugar levels were decreased by insulin. The resynthesis of glycogen occurred during administration of epinephrine which increased blood lactate and sugar but which decreased the glycogen content of the muscles of the control leg (Table I).

Although at first administration of glucose appeared to be of doubtful value, subsequent experiments have shown that it does increase the rate of resynthesis of glycogen definitely but not dramatically. Comparisons of the glycogen following a 1 minute period of work with and without glucose were made.

The mean glycogen for various intervals of rest after 1 minute of work, for animals with glucose and for animals without glucose, is shown in Table II. The mean glycogen content at every juncture of the rest period is greater for the animals with than for those without glucose, but is significant only at 44 and 59 minutes rest. However, the mean difference for the animals with and without glucose for all the periods of rest is 89 ± 26 gm., which is statistically significant. It appears that 29 minutes are too short a time for much accumulation of glycogen and that after rest of more than 1 hour glycogen resynthesis is almost complete in both groups of animals.

TABLE II

Effect of Glucose on Resynthesis of Glycogen after 1 Minute of Work

Time of rest	Without glucose		With glucose		Difference, with glucose minus without glucose
	No. of rats	Mean glycogen	No. of rats	Mean glycogen	
min.		*mg. per 100 gm.*		*mg. per 100 gm.*	*mg. per 100 gm.*
29	9	282 ± 26	3	340 ± 59	58
44	4	360 ± 13	3	460 ± 35	100
59	6	367 ± 20	4	551 ± 30	184
89	4	470 ± 46	3	543 ± 58	73
119	4	446 ± 35	4	478 ± 24	32
Mean					89 ± 26

The figure following ± is the standard error of the mean.

In other experiments one leg was stimulated to contract for 3 minutes and then allowed to rest for 29 or 44 minutes. During the last 3 minutes of the rest period the opposite leg of the same animal was stimulated to contract. The glycogen increase during rest was then calculated as the difference of the glycogen content of the two legs of the same animal. Rats which received 500 to 600 mg. of glucose intraperitoneally or intravenously during the experiments showed a small but definite increase in the rate of resynthesis of glycogen as compared with rats which did not receive glucose (Table III). At the end of 29 minutes of rest the difference between the mean increase of glycogen for the animals with and without glucose was 17 ± 14 mg. The difference is only 1.2 times its standard error, for which ratio the probability

of a chance error is about 10 per cent, so that we could only say at this point that the difference is probably real. However, after 44 minutes of rest the difference between the increase for the animals with and without glucose was 48 ± 21 gm. The difference is now clearly statistically significant. Hines and Knowlton (9) have shown that glucose administered by stomach tube may produce unusually high values of muscle glycogen. After giving glucose in this manner (1.25 gm. 6 and 3 hours before the muscles were removed), we found the glycogen content in the control muscles to vary from 600 to 1000 mg., which range is distinctly higher than the values we found in our other rats.

It is interesting to observe the changes produced by work and rest in animals which have these unusually high amounts of glyco-

Table III

Effect of Glucose on Resynthesis of Glycogen after 3 Minutes of Work

Time of rest	Without glucose		With glucose	
	No. of rats	Mean glycogen increase*	No. of rats	Mean glycogen increase*
min.		*mg. per 100 gm.*		*mg. per 100 gm.*
29	10	47 ± 9	8	64 ± 11
44	10	65 ± 11	11	113 ± 18

The figure after ± is the standard error of the mean.

* Obtained by difference from the control leg of the same animal which was worked 3 minutes at the end of the rest period.

gen in their muscles. As work started, the glycogen broke down rapidly but this process continued much longer than in muscles with ordinary amounts of glycogen. However, the accumulation of lactic acid was not greater and was little, if at all, prolonged. Therefore although utilization of glycogen associated with the starting of work is accompanied by accumulation of lactic acid, subsequent utilization may occur without such accumulation. In a few experiments when after a 1 minute period of work the muscles were allowed to rest for 18 or 28 minutes and then stimulated to work another minute, the starting of work again produced a large accumulation of lactic acid, 214 to 271 mg. This result is similar to that found in rats with ordinary amounts of glycogen but is of greater extent. At the time of removal of the muscles

of the rats which had been given large quantities of glucose by stomach tube the blood sugar varied from 150 to 225 mg. per 100 cc. and the blood lactic acid content from 33 to 69 mg. per 100 cc. Even under these exaggerated conditions the rate of resynthesis of glycogen was still much slower than the rate of breakdown but in some cases was definitely faster than under more normal conditions.

Attempts were made to study the resynthesis of glycogen following the administration of cyanide, iodoacetate, or azide as the sodium salts. Such attempts were ineffective, owing to the general poisoning of the whole animal, which was rapidly followed by death. When these poisons were used in quantities sufficient to prevent continuation of work after the 1st minute, they were without effect on the chemical changes involved in the starting of work, such as the breakdown of glycogen to lactic acid. Under such conditions the effect of these poisons was much the same as was obtained from the removal of the circulation. The glycogen in the control legs was not greatly affected by the poisons, at least over short periods, and the lactic acid content generally increased only 20 to 30 mg. Even when muscles were deprived of their blood supply by ligation of the aorta or amputation of the hind quarters, the glycogen content of control legs did not decrease very rapidly. From 9 to 29 minutes after amputation the lactic acid content of ten control legs varied from 39 to 70 mg. Without circulation the muscles were able to work for about 1 minute only, but they could do this even if they had been without circulation for 20 minutes. During the minute of work glycogen broke down and lactic acid accumulated just as in rats with normal circulation. If muscles which worked 1 minute after amputation were allowed to rest for 15 to 25 minutes and then were stimulated for 1 minute, no work was performed, although an appreciable amount of glycogen was still present.

Comment

Sacks and Sacks (10, 11) have emphasized the importance of differentiating the early or anaerobic phase of work from the subsequent "direct oxidative" phase. It appears that glycogen, although chiefly involved as a source of energy during the anaerobic phase, may under certain conditions also be involved in the oxidative phase. Under our experimental conditions the greatest

part of the muscle glycogen broke down during the 1st minute of work and produced at this time the maximal accumulation of lactic acid and hexose monophosphate. When normal levels of glycogen were available at the beginning of work, a significant decrease in glycogen occurred for 2 minutes after the peak of accumulation of lactic acid had been reached. When unusually large amounts of glycogen were available, the breakdown of glycogen was greatest during the 1st minute and less for the 2nd and 3rd minutes and detectable amounts continued to break down for as long as 30 minutes during continued work. This prolongation of the breakdown of glycogen occurred, however, without any extension of the period of accumulation of lactic acid. The Pasteur enzyme recently described by Stern, Melnick, and DuBois (12) is of considerable interest in this connection. It may be that such a catalyst inhibits the formation of lactic acid from glycogen very soon after work starts but does not prevent subsequent oxidation of small amounts of glycogen for a limited time as work continues.

When muscle glycogen was reduced to minimal levels by 3 minutes of contraction, it was resynthesized so slowly that a rest period of at least 2 hours was required before normal levels were again approached. This great discrepancy between the rates of hydrolysis and resynthesis definitely limits the period of effectiveness of glycogen as a source of energy in muscular contraction. Likewise it eliminates the possibility of explaining the Pasteur reaction on the basis of oxidative resynthesis, as earlier proposed by Meyerhof. Moreover the resynthesis of glycogen in intact, mammalian muscle appears to be quite independent of the presence of lactic acid, since it does not proceed more rapidly when rest ensues after 3 minutes of work than after 15 minutes of work, although the lactic acid content is much higher after the shorter period of work. Also large quantities of lactic acid were administered without any effect on the rate of resynthesis of the glycogen. Resynthesis did not occur without adequate circulation. The rate of resynthesis was increased somewhat by the administration of glucose, although the concentration of this substance in the blood was certainly not the major limiting factor in the rate of deposition of glycogen following muscular activity. The possible importance of the condition of the muscle proteins in this connection cannot be overlooked.

SUMMARY

Breakdown of glycogen in muscles during contractions repeated three times each second is very rapid for 1 minute, less so for the next 2 minutes, and very slow after that. If, however, unusually large amounts of glycogen are present, the time of breakdown is more prolonged. We were unable to modify the breakdown of glycogen and the accumulation of lactic acid in the muscle during the 1st minute of work by the administration of sublethal amounts of cyanide, azide, or iodoacetate, or by depriving the muscle of its blood supply prior to stimulation.

During rest after work of 1 or 3 minutes duration the resynthesis of glycogen is slow. Little glycogen has reformed after 20 minutes, at which time the excess lactic acid has been removed from the muscle. The rate of resynthesis is little influenced by the presence of administered lactate or the administration of epinephrine or by insulin hypoglycemia. The rate of resynthesis is increased somewhat by the administration of glucose.

The discrepancy between the rates of hydrolysis and resynthesis of glycogen definitely limits the effectiveness of glycogen as a source of energy in continued muscular contraction.

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THE EFFECT OF TESTOSTERONE ON THE SERUM LIPIDS OF NORMAL SUBJECTS

By JOSEPH M. LOONEY and ELIJAH B. ROMANOFF

(*From the Memorial Foundation for Neuro-Endocrine Research and the Research Service of the Worcester State Hospital, Worcester*)

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In a recent publication from these laboratories (1) it was shown that injections of testosterone propionate in sesame oil into nine schizophrenic patients produced a significant increase in serum lipids, while injections of sesame oil alone had no effect. In the following paper we wish to present the results of a similar study on fourteen normal male subjects. The subjects received 50 mg. of testosterone propionate[1] daily for 7 days and blood samples were taken before treatment and on the day following the last injection. Serum lipids were determined by the method previously described (2).

Results

The results of the investigation are shown in Table I, and the significance of these values was tested by means of Fisher's technique for small samples.[2] It will be noted that the values before and after medication show no significant change and that except in the case of free cholesterol the values after medication are somewhat lower than those before medication.

[1] We are indebted to the Schering Corporation for the testosterone propionate used.

[2] The following formula was used

$$t = \frac{\bar{d}}{\sqrt{\dfrac{n\Sigma d^2 - (\Sigma d)^2}{n^2\,(n-1)}}}$$

where d = difference between each pair of values, $\bar{d} = \Sigma d/n$ = mean difference.

DISCUSSION

The results of this study are in agreement with the results obtained by Kochakian and his coworkers (3) on normal and castrate dogs. Inasmuch as Randall has shown that the treatment of schizophrenic patients with injections of testosterone propionate (1) produced a significant increase in serum lipid values, the failure to obtain such an increase in our normal subjects is evidence of a disturbed lipid metabolism in such patients.

A fundamental difference between the schizophrenic patient and the normal subject with regard to the metabolism of male sex hormone is shown not only in this action on blood lipids but also in the fact that the effect on the excretion of androgens is entirely

TABLE I

Serum Lipids of Fourteen Normal Subjects before and after Injection of Testosterone

The values are given in mg. per cent.

	Before	After	t, Fisher	P
Total lipid............	535.4	514.4	1.25	>0.20
Phospholipid..........	200.7	190.4	1.84	>0.09
Total cholesterol......	167.8	160.0	0.92	>0.40
Free "	43.2	44.1	0.53	>0.60
Ester "	124.6	115.6	1.04	>0.30

Significance attaches only to values of $P < 0.05$.

different in the two groups. Looney and Howe (4) have shown that, whereas normal subjects react to injections of testosterone propionate by a marked increase in the output of androgens, the patients fail to respond and show no such increase. It would seem probable that these two findings are in some manner interconnected and that the main difficulty is due to an abnormal functioning of the oxidative and reductive functions of the tissues in schizophrenic subjects. This assumption is consistent with previous findings reported from this laboratory that the basal metabolic rate is significantly lower in the patients (5), that the relationship between oxygen intake and carbon dioxide output differs from that of the normal subject (6), and with other studies which point to a defective oxygen metabolism (7).

SUMMARY

The injection of 350 mg. of testosterone propionate during a period of 7 days produced no significant change in the serum lipid values of fourteen normal subjects.

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STEROIDS OF URINE OF OVARIECTOMIZED WOMEN

By H. HIRSCHMANN

(From the Department of Obstetrics and Gynecology and the Gynecean Institute of Gynecologic Research, University of Pennsylvania, Philadelphia)

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It is generally believed that most steroids excreted in urine are derived from substances elaborated in the endocrine glands. In many instances, however, there are definite structural dissimilarities between urinary and glandular steroids and little is known about their metabolic interrelationships. Current views as to the glandular precursors of urinary steroids are based principally on a comparison of their structures and are supported only in a few cases by studies of the excretion products resulting from injected material. As steroid hormones have so far been found in two endocrine glands, the adrenals and the gonads, it seemed of interest to study the effect of the removal of one of these organs on the steroid content of urine. We have, therefore, greatly welcomed an opportunity provided through the cooperation of the staff of the gynecological division of this department to study the urine of ovariectomized women.

The urine of ovariectomized women has already been examined by various investigators using biological and colorimetric methods of assay. The urinary excretion of estrogens (1, 2) and of androgens (2–4) in such cases was lower than that of normal women, while the output of 17-ketosteroids was approximately normal (4). The methods used, however, were not specific for any one excretion product nor applicable to all of the steroids normally found in urine. We have therefore attempted to isolate and to identify the steroids excreted by such patients.

The urine used in this study was pooled from several women on whom a bilateral salpingo-oophorectomy and hysterectomy had been performed. The urine was hydrolyzed with acid, extracted with benzene, and the extract freed of acidic and phenolic com-

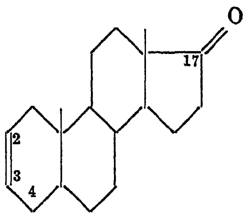
pounds. No attempt was made to isolate the estrogens from the phenolic fraction, since the volume of urine extracted seemed insufficient for this purpose (2). The neutral fraction was separated into ketonic and non-ketonic compounds. Both fractions were further separated into substances precipitable and non-precipitable with digitonin. The ketones which did not precipitate with digitonin were separated by the chromatographic method of Callow (5).

The non-ketonic fraction yielded cholesterol and pregnanediol. From the ketonic fraction five compounds were isolated. Three of these were identified as dehydroisoandrosterone, androsterone, and α-3-hydroxyetiocholanone-17.[1] The two remaining ketones have not been found previously in the urine of women.[2] The first of these new compounds melted at 109°. The analytical figures indicated a formula $C_{19}H_{28}O$. The oxygen atom is present as a carbonyl group. This was suggested by the mode of isolation and confirmed by the formation of a semicarbazone. The location of this carbonyl group at C_{17} was indicated by the characteristics of the light absorption after treatment with *m*-dinitrobenzene and alkali (8). On hydrogenation in the presence of palladium approximately 1 mole of hydrogen was taken up. The reaction product was a ketone with analytical figures corresponding to $C_{19}H_{30}O$. On the basis of its melting point and of a mixed melting point with known material (9) the reduced ketone was identified as androstanone-17. The original ketone was, therefore, an androstenone-17. A compound of this type with similar melting point (104°, uncorrected) and similar crystal form has been obtained by Butenandt and Dannenbaum (9) by treating α-3-chloroandrostanone-17 with potassium acetate at 180°. The position of the ethylenic double bond of this product was not established. Mar-

[1] The isolation of these three ketones from the urine of ovariectomized women has been reported in a preliminary communication (6).

[2] These two compounds may be identical with two as yet unidentified substances melting at 100° and 181° respectively, which have been obtained from the ketonic, non-alcoholic fraction of the urine of normal men by Engel, Thorn, and Lewis (7). Dr. Engel has made a direct comparison of the lower melting substances obtained in the two laboratories. We are indebted to him for the following information. The compound from men's urine melted at 101.5–103° (corrected), that from the urine of ovariectomized women at 107–108.5° (corrected), while a mixture of both melted at 105–107° (corrected). The two substances could not be distinguished by their crystal form.

ker, Kamm, Jones, and Mixon (10) have reported the preparation of Δ_2-androstenone-17 (I) melting at 102° from a 3-chloroandrostanone-17 by treatment with boiling quinoline.



(I)

The assignment of structure for this compound was not based on direct evidence but apparently on the assumption that the removal of hydrogen chloride establishes a double bond between C_2 and C_3 such as occurs in α-3-chlorocholestane (α-cholestyl chloride) when subjected to the same procedure (10). We have treated α-3-chloroandrostanone[3] with pyridine and sodium iodide (employing conditions similar to those used by Pearlman and Wintersteiner (15) in the removal of hydrogen chloride from 7-chloroestrone benzoate) and have obtained an androstenone melting at 110°. If no shift of the double bond occurs during the reaction, the product is Δ_2-androstenone-17 or Δ_3-androstenone-17 or a mixture of both. Its identity with the androstenone isolated from urine was shown by a comparison of the melting points and the rotations of the ketones as well as of the melting points of the semicarbazones. A product of similar melting point (111°), although in lower yield than by the pyridine method, was obtained by treating α-3-chloroandrostanone-17 with boiling quinoline. As this procedure supposedly yields Δ_2-androstenone-17, it is of interest that this reaction product likewise did not depress the melting point of the urinary androstenone. This observation, however, does not seem to provide sufficient evidence for establishing the position of the double bond of urinary

[3] Various starting materials have been used for the synthesis of this compound (9, 11–13). The α-3-chloroandrostanone-17 used in this work was prepared from androsterone and phosphorus pentachloride according to the method of Westphal, Wang, and Hellmann (14). Pearlman and Wintersteiner (15) have demonstrated that a carbonyl group at C_{17} does not react with phosphorus pentachloride under these conditions.

androstenone between C_2 and C_3, since the discrepancies in the melting points of various preparations of androstenone obtained in this laboratory and elsewhere (9, 10) suggest that some or all of these preparations are not homogeneous, but are mixtures most likely of Δ_2- and Δ_3-androstenone. This view is supported by an observation of Wieland, Kraus, Keller, and Ottawa (16) who reported that the thermal dehydration of lithocholic acid yields a mixture of Δ_2- and Δ_3-cholenic acids which cannot be separated by fractional crystallization.

Although the isolation of a urinary steroid that does not carry an oxygen atom at C_3 is no longer a unique finding (17, 18), the question must be raised whether androstenone may not have formed during the course of isolation, especially since an identical product can be prepared from androsterone in the laboratory. The only step which conceivably could have effected such a reaction was the acid hydrolysis of the urine. It seems unlikely that free androsterone (or isoandrosterone) could have yielded androstenone under the conditions employed, but a conjugated form of androsterone may be less stable. Even if androstenone should be shown to be an artifact arising from such a precursor, its isolation from urine in appreciable amounts is not without interest, since similar conditions of hydrolysis are generally used in the determination of urinary androgens and 17-ketosteroids. The effect of such a reaction on the results of bioassays cannot be stated at present, since the androgenic potency of androstenone has not yet been determined. In the colorimetric assay of 17-ketosteroids androstenone is equivalent to an equimolar amount of androsterone.

The second unknown compound found in the ketonic fraction was obtained only in very small amounts and has not been identified. Although it could not be distinguished from androsterone by the absorption spectrum exhibited after treatment with alkaline dinitrobenzene nor by its melting point (184°), a marked depression of the melting point was observed on admixture with androsterone.

Dehydroisoandrosterone (19, 20), androsterone (19, 20), α-3-hydroxyetiocholanone-17 (20), pregnanediol (21, 22), cholesterol (22), and two estranediols (22) have been isolated from urine of normal women. As all of these compounds with the exception of

the estranediols have been obtained also from urine of ovariectomized women, it is evident that in women the excretion of these substances does not require the presence of the ovary. Since the 17-ketosteroids and the pregnanediol found in the urine of ovariectomized women must originate in an extraovarian source (presumably the adrenal cortex), it seems likely that these substances when excreted by normal women are derived at least partly from the same source.

The quantities of the 17-ketosteroids isolated by us are apparently somewhat less than those obtained by Callow and Callow (20) from urine of normal women. An exact comparison is not possible, since the English investigators reported their yields in terms of the crude products. In view of this and in consideration of differences in the isolation procedures employed, of unavoidable losses entailed in the isolation, and of the size of the group of patients studied, it seems questionable whether the differences in yields are significant. However, even a precise knowledge of the quantitative differences in the urinary excretions of these 17-ketosteroids of normal and of ovariectomized women would not allow one to deduce to what an extent the extraovarian source functions in normal women, since the effects of ovariectomy on the secretory activity of the adrenals are still unknown.

The amounts of pregnanediol obtained from the urine of ovariectomized women (about 0.1 mg. per liter) are significantly less than those reported by Venning and Browne (21) for the urine of normal women. The Canadian workers isolated this compound in its conjugated form and found that its excretion depended upon the presence of a corpus luteum. While our findings do not contradict the view that most of the pregnanediol excreted by normal women is derived from the ovary, the data demonstrate that there must be an additional source for this excretion product, most likely the adrenal cortex. This discrepancy between our conclusions and those of Venning and Browne, however, can be explained by the fact that the method for the isolation of the glucuronidate does not allow the estimation of pregnanediol if its concentration is less than 0.6 mg. per liter of urine (23). Since it has been shown that progesterone is converted into pregnanediol in the human (24, 25), and since progesterone has been found in ox adrenals (26), it seems likely that the pregnanediol excreted

in urine of ovariectomized women is derived at least partially from progesterone. As the metabolic conversion of progesterone supposedly yields not only pregnanediol, but also other reduction products, especially allopregnanediol, a search was made for this compound. None, however, has been found.

Current views as to an adrenal origin of the steroids excreted by normal women have been derived mainly from studies of urine of women suffering from tumors or hyperplasia of the adrenal cortex. Since urine of this type contains substances not normally found (27, 28) one is led to suspect that the secretions of these neoplastic or hyperplastic growths may differ from those of normal adrenals not only in amount but also in kind. Nevertheless, the conclusions drawn from our findings agree on the whole with those based on studies of patients afflicted with adrenal disorders. The adrenal origin of dehydroisoandrosterone in normal individuals has been suggested (4), since it was obtained in huge amounts from the urine of patients with tumors of the adrenal cortex (4, 29). Similarly the excretion of abnormally large amounts of pregnanediol in cases of adreno-genital syndrome could be traced at least in one instance to a carcinoma of the adrenal cortex (30). While etiocholanolone (28) has also been found in urine of this type, increased concentrations of androsterone have not been reported. Therefore, an adrenal origin of androsterone in the female is indicated at present only by its excretion in the urine of ovariectomized women.

During the course of this investigation two reports were published dealing with the effects of gonadectomy on the concentrations and nature of urinary steroids in the male. Marker compared the steroid content of the urine of steers (31) and of bulls (32). Estrone, dehydroisoandrosterone, and androsterone were isolated in approximately the same yields from both sources. However, pregnanediol and two allopregnanediols were found in the urine of bulls only. Callow and Callow (33) examined the neutral ketonic fraction of the urine of a eunuch. The normal constituents of this fraction, dehydroisoandrosterone, androsterone, and etiocholanolone, were shown to be present, although in a ratio that differed greatly from that found in urine of normal men (5) or of ovariectomized women. These studies demonstrated that in the male also these neutral 17-ketosteroids cannot be of

purely gonadal origin. The conclusions based on the investigation of the non-ketonic fraction of steers' urine, however, differ from those presented in this report; for in women the excretion of pregnanediol does not depend upon the presence of gonadal tissue.

EXPERIMENTAL[4]

Selection of Patients—Urine was collected from ten ovariectomized women ranging in age from 28 to 44 years. All but one had menstruated regularly up to the time of operation. Otherwise none had shown any signs of an endocrine disturbance. The indication for the operation—a bilateral salpingo-oophorectomy and hysterectomy—was in each instance chronic inflammatory disease of the adnexa. Although the ovaries were found to be the site of adhesions, the surgeons felt certain that the ovarian tissue had been removed completely in each case. Pathological examinations of the ovaries demonstrated the absence of malignant neoplasms. In each case the functional and anatomic criteria for complete ablation of the ovaries were checked at several postoperative examinations and found to be met satisfactorily. The urine collections were not begun until at least 5 weeks after the ovariectomy and were not continued beyond the 7th month after operation. During this time the women were in good health. They did not receive hormonal treatment before or during the collection period with the exception of one woman, who was injected with 20,000 I.U. of amniotin. However, only a small volume of her urine (1.8 liters) had been extracted when this became known. No further collections of urine were made in this case.

Collection and Extraction of Urine—The urine was collected during the winter months in the patients' homes in bottles to which chloroform or benzene had been added as a preservative. The specimens were brought to the laboratory every 2 or 3 days and were stored in a refrigerator until they could be hydrolyzed. No urine was found to be alkaline to litmus. To 10 volumes of urine 1 volume of concentrated hydrochloric acid was added and

[4] All melting points reported are corrected. The microanalyses were carried out by Mr. William Saschek (College of Physicians and Surgeons, New York), the colorimetric measurements by Mrs. D. Leekley.

the mixture was refluxed for 20 to 30 minutes. The hydrolyzed urine in batches of 3.8 liters was extracted with benzene in a continuous extractor[5] for 4.5 to 7 hours. Hydrolysis and extraction were completed within 2 to 4 days after the receipt of the urine.

Fractionation of Extracts—The benzene extracts were combined periodically, were washed with dilute sodium bicarbonate solution and with water, and were taken to dryness *in vacuo*. The benzene residues obtained from 250 liters of urine were distributed between 3.5 liters of ether and 1 liter of 2 per cent sodium hydroxide solution. The ether phase was washed six times with 400 cc. of alkali and repeatedly with water and then taken to dryness. The residue weighed 7.99 gm. (Batch I). The neutral fraction obtained from another 260 liters of urine yielded 9.59 gm. (Batch II). Both neutral fractions were separated into ketonic and non-ketonic material by means of Reagent T (15 gm.) of Girard and Sandulesco (34). The ketonic fractions contained 2.54 and 2.92 gm., the non-ketonic 4.40 and 5.17 gm. respectively. In the following sections the fractionation of Batch I is described; unless stated otherwise all yields refer, therefore, to an extract of 250 liters of urine.

The ketonic fraction (2.54 gm.) was dissolved in 27 cc. of 75 per cent ethanol. A solution of 400 mg. of digitonin in 13 cc. of 75 per cent ethanol was added. After this had stood for a day the digitonide was collected and washed with 6 cc. of cold 75 per cent alcohol and repeatedly with dry ether. It was dissolved in 2.5 cc. of pyridine. 25 cc. of dry ether were added. The digitonin was removed and thoroughly extracted with ether. The combined ether solutions were washed with dilute hydrochloric acid and with water and yielded 64.7 mg. (ketones precipitable with digitonin). The supernatant and the washings of the digitonide were taken to dryness and repeatedly extracted with ether. The ethereal solutions yielded 2.311 gm. (ketones non-precipitable with digitonin).

Ketones Precipitable with Digitonin; Dehydroisoandrosterone—The digitonin-precipitable fraction (64.7 mg.) was leached with 30 cc. of warm carbon tetrachloride (Eastman), which dissolved 62.4 mg. The solution was passed through a column (160 ×

[5] The extractor used was similar to one designed by Professor F. C. Koch, who very kindly sent us a blue-print of his model.

16 mm.) of Brockmann's aluminum oxide. The colored impurities were separated into a brown, a purple, and a yellow zone. The column was washed with 150 cc. of carbon tetrachloride, with 300 cc. of carbon tetrachloride containing 0.1 per cent ethanol, and with 275 cc. of carbon tetrachloride containing 0.2 per cent ethanol. These solvents eluted 1.6, 6.2, and 2.7 mg. respectively. At this point the yellow zone had reached the bottom of the column. Subsequent washings with 175 cc. of carbon tetrachloride containing 0.2 per cent ethanol and twice with 150 cc. of carbon tetrachloride containing 0.4 per cent ethanol yielded 22.0, 14.6, and 3.1 mg. respectively (Fractions *a*, *b*, *c*). Finally 325 cc. of carbon tetrachloride containing 1 per cent alcohol eluted 4.1 mg.; this eluate contained the purple pigment.

Fractions *b* and *c* were combined and recrystallized from 80 per cent methanol. 12.2 mg. of colorless needles were obtained, melting at 141–151°. Fraction *a* yielded 15.7 mg. of a less pure product melting at 133–148°. For preparing derivatives both preparations of dehydroisoandrosterone were combined.

Dehydroisoandrosterone Benzoate—0.03 cc. of benzoyl chloride was added to a solution of 11.7 mg. of dehydroisoandrosterone in 1 cc. of pyridine. After standing for 12 hours at room temperature, the benzoate was precipitated by the addition of water. The precipitate was removed by centrifuging and repeatedly washed with water. The reaction product showed a characteristic low solubility in acetone and was recrystallized four times from this solvent. The benzoate melted at 247–251°. A mixture with an authentic specimen melting at 254–256.5° melted at 250–256.5°.

Analysis—$C_{26}H_{32}O_3$.	Calculated.	C 79.55, H 8.22
	Found.	" 79.18, " 8.39

Dehydroisoandrosterone Acetate—11.2 mg. of dehydroisoandrosterone were dissolved in 0.6 cc. of pyridine and 0.3 cc. of acetic anhydride and kept at room temperature for 12 hours. The acetate was precipitated by gradual addition of cold water. The crude product (11.7 mg.) melted at 143–147°. Even after ten recrystallizations (eight from dilute methanol, two from dilute acetone) the melting point had not become sharp or constant. However, the melting point of the final product (161–164°) was

not depressed by admixture with dehydroisoandrosterone acetate which had been prepared from cholesterol (35).

Fractionation of Ketones Non-Precipitable with Digitonin—Since the chromatographic separation of 2.3 gm. of ketones according to Callow (5) would have required considerable amounts of solvent, an attempt was made to effect the separation of androsterone and α-3-hydroxyetiocholanone-17 over a relatively shorter column. It appears that this modification is satisfactory for preparatory purposes and that after such treatment one recrystallization suffices for obtaining fractions of approximately the same degree of purity as those obtained directly by the original method when applied to the neutral ketones from the urine of normal women (20).

The ketones non-precipitable with digitonin were dissolved in 40 cc. of carbon tetrachloride and passed through a column (290 × 20 mm.) of Brockmann's aluminum oxide. The column was eluted with carbon tetrachloride to which increasing amounts of absolute alcohol had been added. The volumes and compositions of the eluants and the weights and properties of the eluates are given in Table I. After Fraction 12 had been collected, the column was cut into seven parts which were eluted separately with methanol, starting with the lowest zone of the column. These eluates are also listed in Table I (Fractions 13 to 19).

Isolation of Androstenone-17—Fraction 1 failed to crystallize. In order to secure more of the ketone isolated from Fraction 2, Fraction 1 was subjected to another adsorption procedure. The material, which had been stored for several months, was leached with 25 cc. of carbon tetrachloride. 25 mg. of an amorphous product remained undissolved and were removed by filtration. The filtrate was passed through a column (195 × 20 mm.) of aluminum oxide. The adsorbent was washed with three successive portions of 100 cc. of carbon tetrachloride, which eluted 2 mg. and 8 mg. of resinous material and 34 mg. of a crystalline product (Fraction 1a) respectively. Further elution with carbon tetrachloride (150 and 250 cc.) and with carbon tetrachloride containing 0.2 per cent ethanol (470 cc.) yielded 3.8, 2.8, and 8.7 mg. of semicrystalline products. By continued elution only colored resinous material was obtained. Fraction 1a was recrystallized twice from dilute methanol. 20.9 mg. of colorless rectangular plates were obtained, melting at 107–109°. The melting point could not be

raised by further recrystallization. Admixture with specimens of androstenone prepared from α-3-chloroandrostanone by the pyridine method (melting at 110° and 114.5° respectively) or by the

TABLE I

Chromatographic Analysis of Ketones Non-Precipitable with Digitonin

Fraction No.	Eluant (CCl_4) Volume	Eluant (CCl_4) Ethanol content	Eluate Crude weight	Eluate Once recrystallized Weight	Eluate Once recrystallized M.p.	Compound isolated
	cc.	per cent	mg.	mg.	°C.	
1	340	0	164*			Androstenone-17
2	180	0	24†			Ketone melting at 184°
3	250	0.1	50*			
	375	0.2				
4	130	0.2	133*			
5	50	0.2	96*			
6	190	0.2	278‡	121	143–169	Androsterone (6–8)
7	200	0.2	162‡	142 (7–8)	153–177	
8	200	0.2	78‡			
9	200	0.2	72*			
10	200	0.2	80‡	51	123–136	α-3-Hydroxyetiocholanone-17 (10–12)
11	200	0.2	88‡	135 (11–12)	131–141	
12	250	0.2	93‡			
	Height of zone eluted	Eluant				
	cm.					
13	3.8	Methanol	111‡	113 (13–14)	131–141	α-3-Hydroxyetiocholanone-17 (13–15)
14	1.9	"	52‡			
15	1.9	"	36†			
16	3.8	"	49*			
17	7.8	"	107*			
18	8.0	"	232*			
19	1.8	"	97*			

* Resinous.
† Partly crystalline.
‡ Crystalline.

quinoline method (10) (melting at 111°) did not depress the melting point.

Analysis—$C_{19}H_{28}O$. Calculated. C 83.77, H 10.36
Found. " 83.63, " 10.24

Rotation—$[\alpha]_D^{28} = +152°$ (0.5% in 95% ethanol)

On treatment with alkaline dinitrobenzene a purple pigment formed. Data on its absorption characteristics are given in Table II.

The ketonic fraction derived from Batch II yielded on similar treatment 36 mg. of rectangular plates melting at 96–102°. On further recrystallization the melting point was raised to 104.5° (the sample softened at about 93°). Admixture with androstenone obtained from Batch I did not depress the melting point. This material was used for preparing androstenone semicarbazone and androstanone.

Androstenone-17 Semicarbazone—A solution of 9 mg. of androstenone, 30 mg. of semicarbazide acetate, and 4 mg. of potassium

TABLE II

Extinction of Pigments Formed with Alkaline m-Dinitrobenzene (Zimmermann Reaction)

Compound	Amount	Extinction at				
		400 mμ	420 mμ	520 mμ	540 mμ	660 mμ
	γ					
Androstenone-17.	60	0.20	0.25	0.61	0.55	0.08
Ketone melting at 184°.	64	0.18	0.22	0.61	0.55	0.07
Androsterone.	64	0.17	0.22	0.59	0.53	0.07

The reaction was carried out as described by Callow *et al.* (8). The extinction (−log T) was determined with an Evelyn photoelectric colorimeter with filters with maxima of transmission at the wave-lengths indicated in the table.

acetate in 1 cc. of 90 per cent ethyl alcohol was heated under a reflux on a steam bath. A precipitate formed within a few minutes. After 1.5 hours the mixture was allowed to cool and a small volume of water was added. The semicarbazone was separated by centrifuging and thoroughly washed with water. The crude product (9.8 mg.) was very sparingly soluble in ethanol. It was dissolved in glacial acetic acid and precipitated by the addition of ethanol. The final product melted with decomposition at a temperature that varied appreciably with the speed with which the sample was heated. The melting points which were observed on various determinations ranged from 283° to 295°. Androstenone semicarbazone prepared from synthetic androstenone-17 showed

the same behavior. There was no depression ot the melting points when the two preparations were mixed.

Analysis—(Sample dried at 110° *in vacuo*)
$C_{20}H_{31}N_3O$. Calculated, N 12.76; found, N 12.12

Androstanone-17—16 mg. of androstenone, 425 mg. of reduced palladiumized calcium carbonate (1 per cent) (36), and 11 cc. of ethanol were shaken in an atmosphere of hydrogen until the reaction ceased (15 minutes). 1.3 cc. of hydrogen (27°, 761 mm. of Hg) were taken up. The reaction product was fractionated by means of Girard's Reagent T. The ketonic material (15 mg.) thus obtained was recrystallized from dilute acetone and yielded 13 mg. of hexagonal platelets melting at 121–122°. Admixture with a specimen of androstanone-17 (melting at 121–122°) obtained by catalytic reduction of synthetic androstenone-17 did not depress the melting point.

Analysis—$C_{19}H_{30}O$.	Calculated.	C 83.15, H 11.02
	Found.	" 82.76, " 10.82

Preparation of Androstenone-17—51 mg. of α-3-chloroandrostanone-17 melting at 172–174°, 400 mg. of sodium iodide, and 2 cc. of pyridine were refluxed for 40 hours under anhydrous conditions. The reaction mixture was distributed between ether and water. The ether was washed with dilute sodium sulfite solution, with dilute hydrochloric acid, and with water. The ether yielded 25 mg. of almost colorless crystals. Upon recrystallization from methanol, dilute methanol, and dilute acetone 19 mg. of rectangular platelets were obtained that showed a constant melting point of 107.5–110°. Rotation, $[\alpha]_D^{28} = +151°$ (0.8 per cent in 95 per cent ethyl alcohol).

Another batch of androstenone prepared by the same method melted at 111–114.5°. The melting point could not be changed by further recrystallization.

Analysis—$C_{19}H_{28}O$.	Calculated.	C 83.77, H 10.36
	Found.	" 83.96, " 10.21

The lower melting preparation was used for obtaining reference specimens of androstenone semicarbazone and of androstanone.

Ketone Melting at 184°—Fraction 2 was sublimed in a high vacuum (about 10^{-4} mm. of Hg) at 80°. The sublimate (5.6 mg.)

was recrystallized three times from methanol. 1 mg. of colorless prisms was obtained, melting at 183–184°. A mixture with androsterone melted at 148–165°. An approximate absorption spectrum of the pigment produced on treatment with *m*-dinitrobenzene and potassium hydroxide is given in Table II.

Androsterone—Fractions 7 and 8 were combined and recrystallized four times from aqueous ethyl alcohol. 100 mg. of colorless prisms were obtained, melting at 182–183°. No change in melting point was observed when a sample was mixed with an authentic specimen of androsterone melting at 182–183°.

Analysis—$C_{19}H_{30}O_2$. Calculated. C 78.57, H 10.41
Found. " 78.64, " 10.46

Rotation—$[\alpha]_D^{20} = +97°$ (1% in ethanol)

Fraction 6 was recrystallized from dilute alcohol and from the last three mother liquors obtained in purifying Fractions 7 and 8. 97 mg. of androsterone were obtained, melting at 175–178°. A further crop (21 mg.) of crystals with the same melting point was isolated from the mother liquors by high vacuum distillation and recrystallization. The identity of these preparations was confirmed by mixed melting point determinations with androsterone.

Androsterone Benzoate—A pyridine solution of androsterone melting at 183° was treated for 12 hours with benzoyl chloride at room temperature. The mixture was diluted with water and taken up in benzene. The benzene layer was washed with sodium carbonate, with hydrochloric acid, and with water and was taken to dryness. The benzoate was recrystallized from absolute alcohol. The final product, an authentic specimen of androsterone benzoate, and a mixture of both all melted at 178–179°.

α-3-Chloroandrostanone-17—109 mg. of androsterone melting at 177–181° were dissolved in 8 cc. of chloroform. 110 mg. of dry calcium carbonate were suspended in the solution. The stoppered reaction vessel was placed in an ice bath and kept agitated while 156 mg. of phosphorus pentachloride were added in small portions during a period of 35 minutes. After 10 more minutes of shaking, 10 cc. of a cold 7 per cent sodium bicarbonate solution were added. The mixture was extracted with benzene, and the aqueous phase was separated, acidified, and extracted with benzene. The combined benzene extracts were washed with dilute hydrochloric acid,

with sodium bicarbonate solution, and with water, and yielded on evaporation 114 mg. of crystalline material. Upon recrystallization from alcohol 62 mg. of colorless needles were obtained, melting at 172–174°. The mother liquors yielded another 9 mg. melting at 170–172°.

Analysis—$C_{19}H_{29}ClO$. Calculated. C 73.88, H 9.46
Found. " 73.78, " 9.05

α-3-Hydroxyetiocholanone-17—Fractions 11 and 12 were combined and were recrystallized five times from aqueous alcohol. This product (94 mg.) melted at 145–146.5° and was still slightly pigmented. Colorless needles melting at 144–147° were obtained upon recrystallization from a mixture of benzene and petroleum ether (about 1:1).

Analysis—$C_{19}H_{30}O_2$. Calculated. C 78.57, H 10.41
Found. " 78.72, " 10.57

Rotation—$[\alpha]_D^{28} = +109°$ (1% in ethanol)

The second to fifth mother liquors of Fractions 11 and 12 were combined with Fraction 10 that had been recrystallized once. On recrystallization 62 mg. of etiocholanolone were obtained, melting at 144–147.5°. Fractions 13 and 14 were freed from an impurity that was only sparingly soluble in absolute alcohol and yielded 96 mg. on recrystallization from dilute alcohol. This product melted at 142–151°. Fraction 15 was combined with the mother liquors obtained in the purification of the preceding fractions and distilled in a high vacuum at 150°. The distillate after recrystallizations from dilute acetone and from a mixture of benzene and petroleum ether yielded another 31 mg. of etiocholanolone melting at 139–149°. There was no depression of the melting points when the various preparations of α-3-hydroxyetiocholanone-17 were mixed with each other.

α-3-Hydroxyetiocholanone-17 Benzoate—19 mg. of etiocholanolone (melting at 147°) were dissolved in 1 cc. of pyridine and were treated with 0.06 cc. of benzoyl chloride at room temperature. The benzoate upon several recrystallizations from absolute alcohol melted at 163–164.5°.

Analysis—$C_{26}H_{34}O_3$. Calculated. C 79.14, H 8.69
Found. " 79.13, " 8.97
" 78.93, " 8.54

α-3-Hydroxyetiocholanone-17 Acetate—18.2 mg. of etiocholanolone were acetylated in the usual manner. The crude product (21.4 mg.) could be obtained in crystalline state only after it had stood for several weeks in the ice box. When seeding crystals were available, no difficulty was encountered in recrystallizing this material from dilute methanol. The acetate melted at 93–95°.

Fractionation of Non-Ketonic Material—50 cc. of 95 per cent alcohol were added to the non-ketonic fraction obtained from Batch I (4.40 gm.). The mixture was brought to a boil and kept at room temperature overnight. The dark residue which failed to dissolve was removed by centrifuging and was washed with 25 cc. of 80 per cent alcohol. To the combined supernatants a solution of 975 mg. of digitonin in 25 cc. of 90 per cent alcohol was added. A digitonide precipitated and was separated after 40 hours standing at room temperature. It was washed with 25 cc. of cold 90 per cent alcohol and six times with ether, and was decomposed in the usual manner. The digitonin-precipitable fraction (196 mg.) crystallized readily. The mother liquor and the washings of the digitonide were combined, taken to dryness, and repeatedly extracted with ether. The extract yielded 3729 mg. of a dark resin (non-ketonic fraction non-precipitable with digitonin).

Non-Ketonic Fraction Precipitable with Digitonin; Cholesterol—The non-ketonic digitonin-precipitable fraction (196 mg.) was recrystallized from 95 per cent ethanol, from methanol, and from petroleum ether. The resulting crystals (92 mg.) melted at 147–149°. A mixed melting point with authentic cholesterol showed no depression. The mother liquors yielded another 37 mg. of cholesterol melting at 145–147°. An acetate was prepared which melted at 114–115°. Its melting point remained unchanged upon admixture with an authentic specimen of cholesterol acetate.

Non-Ketonic Fraction Non-Precipitable with Digitonin; Pregnanediol—The non-ketonic fraction which did not precipitate with digitonin (3729 mg.) was taken up in carbon tetrachloride and passed through a column (200 × 30 mm.) of aluminum oxide. The column was eluted with 2.4 liters of carbon tetrachloride and 1.3 liters of carbon tetrachloride containing 10 to 30 per cent of ether. These eluants developed a chromatogram exhibiting a great number of distinct colored zones. However, the seventeen

fractions that were collected during the elution failed to crystallize and their description is therefore omitted. The column was then washed with approximately 500 cc. of methanol which yielded 1.909 gm. of resinous material. This residue was dissolved in a small volume of acetone. An amorphous precipitate formed which proved to be insoluble in ethanol. It was discarded. After several days a crystalline precipitate formed in the acetone mother liquor. The precipitate was washed with cold acetone and freed from material insoluble in alcohol. After recrystallization from acetone and from dilute alcohol the product (28.6 mg.) melted at 235–237°. A mixture with a sample of pregnanediol prepared from sodium pregnanediol glucuronidate melted at 235.5–238°.

23.5 mg. of pregnanediol were dissolved with warming in 2 cc. of pyridine and 1.2 cc. of acetic anhydride and kept at room temperature for 15 hours. The excess of acetic anhydride was hydrolyzed by the addition of water. The acetate was isolated in the usual manner and recrystallized from methanol. It was obtained in two modifications, one melting at 165–166°[6] and the other at 179.5–180°. Both modifications when allowed to solidify after fusion usually melted at 166°. A mixture of the high melting modification (m.p. 177.5–179.5°) with an authentic specimen of pregnanediol diacetate melting at 179.5–180.5° melted at 179–180.5°. When the specimens were remelted, the melting points were 165°, 166°, and 166° respectively.

Analysis—$C_{25}H_{40}O_4$.	Calculated.	C 74.21, H 9.97
	Found.	" 73.65, " 9.92

Similar amounts of pregnanediol were obtained from the non-ketonic fraction of Batch II by a less involved procedure. The material was freed from pigments insoluble in 95 per cent alcohol (70 cc.) and distilled in a high vacuum for 21 hours while the temperature was raised to 150°. The distillate (2.7 gm.) was dissolved in 5 cc. of acetone. On chilling, a semicrystalline precipitate (117 mg.) formed which was separated. The supernatant was concentrated and seeded with allopregnanediol.[7] No further pre-

[6] Preparations of pregnanediol diacetate with a similar melting point have been described previously (37, 27).

[7] Allopregnanediol was obtained through the courtesy of Dr. O. Kamm of Parke, Davis and Company.

cipitate formed even when the solution was kept at −12°. The precipitate was repeatedly recrystallized from acetone. The final product melted at 226–232°. It was acetylated and yielded after several recrystallizations 21 mg. of pregnanediol diacetate melting at 177.5–179°. The mother liquors were combined with the acetylated mother liquors of the free diol. The material (97 mg.) was taken up in petroleum ether and adsorbed on alumina. Fractional elution with the same solvent and with petroleum ether containing 5 to 20 per cent of benzene yielded two distinct crystalline fractions in addition to small amounts of resinous products. The crystalline material which was eluted more readily (10.8 mg.) was recrystallized from methanol. The final product (2.6 mg.), which presumably still contained impurities, melted at 104–109°. It has not been identified.

Analysis—$C_{22}H_{34}O_4$.	Calculated.	C 72.89,	H 9.45
$C_{23}H_{36}O_4$.	"	" 73.36,	" 9.64
	Found.	" 72.94,	" 9.94

The other crystalline fraction (28 mg.) was obtained from three eluates which all melted above 150°. It yielded upon recrystallization 13 mg. of pregnanediol diacetate melting at 176–179°. When a synthetic mixture of pregnanediol diacetate and allopregnanediol diacetate[7] was subjected to the same chromatographic procedure, it was found that allopregnanediol diacetate was less readily eluted than its stereoisomer. The data obtained seem to indicate that the crude specimen of pregnanediol that was isolated did not contain appreciable amounts of allopregnanediol.

SUMMARY

Dehydroisoandrosterone, androsterone, α-3-hydroxyetiocholanone-17, pregnanediol, and cholesterol have been obtained from the urine of ovariectomized women.

In addition two other 17-ketosteroids have been isolated, one of which has been shown to be identical with a preparation of androstenone-17 obtained from α-3-chloroandrostanone-17.

The preparation α-3-chloroandrostanone-17 from androsterone is described.

It is suggested that in women dehydroisoandrosterone, androsterone, α-3-hydroxyetiocholanone-17, and pregnanediol are derived

at least partially from substances that are formed in the adrenal cortex.

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KETOGENIC ACTION OF ODD NUMBERED CARBON FATTY ACIDS

By EATON M. MacKAY, ARNE N. WICK, and CYRUS P. BARNUM

(*From the Scripps Metabolic Clinic, La Jolla, California*)

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The statement is commonly made that unlike even numbered carbon fatty acids the odd numbered carbon fatty acids do not give rise to acetone bodies in the mammalian organism. It was shown long ago that propionic acid (1) and its higher homologues valeric and heptylic acids (2) could form glucose in the phlorhizinized organism. This led to the conclusion (3) that higher fatty acids with uneven numbers of carbon atoms yielded glucose in so far as they might form propionic acid by β oxidation. This glycogenic action of the odd numbered carbon fatty acids has more recently been demonstrated in a very convincing manner by Deuel *et al.* (4), who showed a consistent increase in the hepatic glycogen of rats after feeding propionic, valeric, heptylic, and pelargonic acids. Since the levels of liver glycogen were in the same range, they concluded "that β oxidation proceeds in a relatively quantitative manner with fatty acids having an odd number of carbon atoms." Since β oxidation of the even numbered carbon fatty acids gives rise (5) to acetone body formation, why should it not do so from the odd numbered carbon fatty acids. In other words what is the fate of the carbon atoms removed during their oxidation to the 3-carbon glucose-forming moiety? Since the odd numbered carbon fatty acids may form glycogen, it has been difficult to conceive of them as forming acetone bodies at the same time. However, the recent observation that short chain even numbered carbon fatty acids may give rise to acetone bodies in the presence of large amounts of liver glycogen and during the formation of glycogen by other compounds (6) makes this possibility less unreasonable. The question with which our experiments are concerned is whether odd numbered carbon fatty

acids higher than propionic may form acetone bodies as well as glycogen.

Methods

The odd numbered carbon atom fatty acids are rather toxic. The ethyl and glycerol esters are less toxic in the order named. Both esters were used, depending on their availability. Experiments on rats showed that the absorption of these fatty acids is

TABLE I

Details of the Experiments Shown in Fig. 1

Experiment No.	Weight of rabbit*	Fatty acid† fed	Doses of fatty acid fed: As fatty acid	Doses of fatty acid fed: As acetone bodies‡	Liver glycogen at end of experiment
	kg.		*mM per kg.*	*mM per kg.*	*per cent*
1	1.6	C_3, propionic	99.5, 0 hr.; 99.5, 2 hrs.	0.0	3.8
2	3.0	C_5, valeric	25.6, 0 " ; 25.6, 4 "	25.6	3.2
3	1.8	" "	48.8, 0 " ; 48.8, 2 "	48.8	8.5
4	2.1	C_7, heptylic	33.8, 0 " ; 33.8, 3 "	67.6	7.7
5	1.8	" "	37.2, 0 " ; 37.2, 4 "	74.4	8.9
6	2.4	C_9, pelargonic	22.4, 0 " ; 22.4, 3 "	67.2	7.5
7	2.2	" "	22.4, 0 " ; 11.2, 3 "	50.4	4.2
8	3.0	C_{11}, undecylic	14.8, 0 " ; 14.8, 2 "	59.2	5.9

* Female rabbits were used except in Experiment 3.

† The fatty acids were fed in the form of the ethyl esters in Experiments 2, 6, 7, and 8 and in the form of the glycerol esters in the other experiments.

‡ Calculated on the assumption that all of the carbon atoms over 3 of all these fatty acids are capable of conversion to acetone bodies.

very slow in comparison with even numbered carbon fatty acids in whatever form they are fed. For this reason observations were extended over a considerable period of time. Fully fed rabbits were used. The esters were administered by stomach tube and at varying intervals thereafter arterial blood specimens were drawn from the heart. Acetone bodies were determined on the blood specimens by the method of Barnes and Wick (7) and the liver glycogen concentration was determined (8) at the end of each experiment. Typical results are presented in Table I and Fig. 1.

Results

The data presented here show that propionic acid (C_3) does not give rise to acetone bodies. All of the other fatty acids examined (C_5 to C_{11}) proved to be ketogenic. Although not strictly comparable, the doses of fatty acids administered were of the same order of magnitude. The variations in the blood acetone body curves are probably due chiefly to differences in absorption rates.

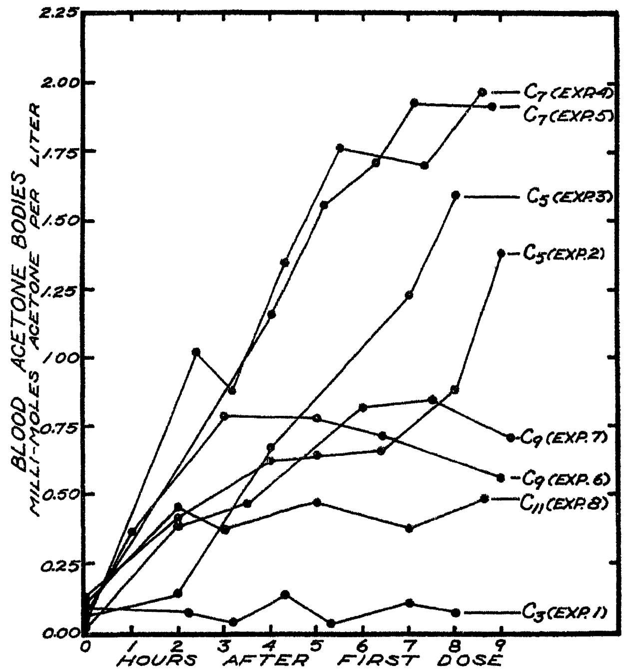

FIG. 1. Effect of feeding ester of odd numbered carbon fatty acids on blood acetone body concentration of fed rabbits.

Undecylic (C_{11}) acid for instance is absorbed at a much lower rate in terms of mM than heptylic acid in the rat.

DISCUSSION

From the data presented here we have evidence for the formation of acetone bodies from fatty acids, which may simultaneously form glycogen.

Embden (9) found that the perfusion of a liver with fatty

acids containing an odd number of carbon atoms in the chain did not increase the amount of acetoacetic acid in the perfusate. There are many possible explanations for this, the most likely one being the failure to use or being able to use a concentration of fatty acid high enough in terms of possible acetone formation to give a measurable increase. It is almost impossible to demonstrate the ketogenic activity of the odd numbered fatty acids through the slow intravenous injection of their sodium salts in the intact animal. Their toxicity makes it impossible to inject them at a high enough rate.

Butts (10), Deuel (4), and coworkers reached the conclusion that the C_9, C_7, and C_3 acids were not ketogenic and that (C_5) valeric acid was only slightly so, if at all. The explanation of their results rests on the fact that the conclusions were based on observations of the ketonuria, and the glycogen-forming propensity of these acids would so reduce or abolish the acetone body formation from endogenous sources as to make ketonuria observations of little value. The acetone bodies have a rather high renal threshold and, without their formation from endogenous sources, it is almost impossible to administer enough of any ketogenic substance which is ketogenic in the presence of adequate carbohydrate stores to raise the blood acetone body level high enough for the production of a measurable ketonuria.

The odd chain fatty acids have the peculiar property of being antiketogenic in so far as the production of acetone bodies from endogenous sources is concerned but are ketogenic (except for propionic) when their own fate is considered.

Confirmation of our results in the intact animal is given by the observations of Jowett and Quastel (11) with liver slices. They found that acetoacetic acid was produced by C_9, C_7, and C_5 acids but not by (C_3) propionic acid.

The fact that valeric acid yields acetoacetic acid is strong evidence in favor of the β oxidation-acetic acid condensation hypothesis of acetone body formation from fatty acids (5), for 3 of the 5 carbon atoms of valeric acid presumably go to form glycogen (2, 4).

SUMMARY

In fed rabbits with adequate amounts of liver glycogen and without a ketosis the feeding of odd numbered carbon fatty

acids higher than propionic in the form of their ethyl or glycerol esters leads to an increase in the level of acetone bodies in the blood. Valeric, heptylic, pelargonic, and undecylic acids were all ketogenic.

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MANOMETRIC CARBON DETERMINATION

By DONALD D. VAN SLYKE and JORDI FOLCH

With the Assistance of John Plazin

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

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The method here described depends on combustion in a mixture of chromic, iodic, sulfuric, and phosphoric acids. With all types of organic substances thus far tried this mixture gives theoretical yields of carbon dioxide with 1 to 3 minutes boiling. The CO_2 is absorbed in alkali solution in the Van Slyke-Neill manometric apparatus and then determined by a development, modified in most details, of the procedure introduced by Backlin (1) and refined by Van Slyke, Page, and Kirk (13). For absorption of the CO_2 a solution of NaOH and hydrazine is used, the hydrazine serving to reduce any halogens evolved during the combustion.

No changes in the present method are required by the presence of nitrogen, sulfur, halogen, alkali metals, nor, so far as has been ascertained, any other of the substances that interfere with the usual forms of the dry combustion (6).

The same apparatus serves for *submicroanalyses* with 0.3 to 0.7 mg. of carbon, for *microanalyses* with 2 to 3.5 mg., and for *macroanalyses* with 8 to 15 mg. In the submicroanalyses the mean error is of the order of 1 part in 200, while in the micro and macro it is of the order of 1 in 500. The time routinely required for an analysis, after the sample has been weighed or measured, is about 15 minutes. For precise carbon determinations to identify organic compounds in the writers' laboratory the method has replaced the dry combustion.

For analyses of pure compounds wet combustion methods have hitherto never attained acceptance by the organic chemist, although for many years such combustions have been used for exact or approximate carbon determinations in special materials.

Past failure of the wet combustion to find a place in the organic laboratory appears due to the fact that the oxidizing solutions used did not give completely quantitative results with the more difficultly combustible compounds. The chromic acid oxidizing mixture of Friedemann and Kendall (4), for example, gave 99 to 100 per cent of theoretical CO_2 yields with a series of twenty organic substances, except stearic acid and carbazole, with which the yield was 98 to 99 per cent. The chromic acid mixture used by Van Slyke, Page, and Kirk (13) was similar to that of Friedemann and Kendall, except that it contained less water and was for that reason somewhat more vigorous in oxidation. Iodic acid, introduced by Strebinger (10), and used by Stanek and Nemes (9) and by Christensen and Facer (2), gives theoretical yields of CO_2 with some compounds, but is slower than chromic acid in its action. In the experience of the writers, iodic acid, even more than chromic, falls short of quantitative results with some of the difficultly combustible substances; also it has shown such unpredictable variations as to yield a theoretical carbon with tyrosine, but only 70 per cent with tryptophane. It is accordingly not surprising that the only wet combustion sanctioned for elementary carbon determination by Meyer (6) is one in which the gases freed by chromic acid oxidation are passed through a regular combustion tube to assure complete oxidation. Since this procedure combines the complications of the two types of combustion, Meyer naturally recommends it only for materials which resist analysis by the dry combustion.

In arriving at the present combustion mixture, catalysts such as silver salts (7) and ceric salts (5) used by previous analysts with the wet combustion were added to chromic acid mixtures in analyses of such difficultly oxidizable substances as cholesterol, palmitic acid, and pentaacetyl glucose, and were found to be without benefit. The CO_2 yields remained below the theoretical. Various other catalytic and oxidizing adjuvants were tried with entirely negative results. The only exception was iodic acid which, added to chromic acid, did increase the yields. Possibly the effect is due to the specific ability of iodic acid to oxidize CO to CO_2. The low yields of CO_2 with former chromic acid methods have been attributed to the escape of some of the carbon in the form of CO (3). In studies of the effect of water content on the

completeness of combustion it was furthermore found, in accord with Friedemann and Kendall (4), that the more anhydrous mixtures burned more rapidly and completely. Hence the present mixture was evolved, which is a solution of chromic and iodic acids in a nearly anhydrous medium of sulfuric and phosphoric acids.

So few details of the apparatus, reagents, and manipulation of Van Slyke, Page, and Kirk (13) remain unchanged that it has proved simpler and more concise to describe the present procedure completely, rather than to attempt to refer the reader to those details of the original that remain unaltered.

MICRO- AND SUBMICROCOMBUSTIONS

Apparatus[1]

The apparatus used for the combustion is shown in Figs. 1 and 2. With changes in details it is the same used by Van Slyke, Page, and Kirk (13). The combustion tube, *T*, and the connecting tube *Q* are made of Pyrex glass. The core of the cock below *F* must also be of Pyrex glass, or unequal expansion when the apparatus warms in use may cause the cock to stick.

Accessory apparatus is shown in Figs. 3 and 4.

In Fig. 5 is shown the construction of a sheet aluminum scoop and counterpoise that have proved convenient for weighing samples on the microbalance. For handling the scoop one uses small crucible tongs with chamois skin glued to the inside of the jaws. If the scoop were handled with unprotected metal jaws, the aluminum would be worn off at a rate which would rather rapidly diminish its weight.

Alundum (crystalline alumina) pieces to promote smooth boiling of the combustion mixture are cleaned by boiling them in combustion fluid, washing with water, and drying. They are kept protected from dust in a small Petri dish. The pieces, 1 to 2 mm. long, are size No. 14 of the Norton Company. Presumably sand of similar particle size would do as well.

Reagents—

Chromic acid combustion fluid. In a 1 liter Pyrex Erlenmeyer flask, provided with a ground glass stopper, place 25 gm. of CrO_3,

[1] The apparatus can be obtained from Eimer and Amend, and from E. Machlett and Son, New York.

followed by 167 cc. of syrupy phosphoric acid (sp. gr. 1.7) and 333 cc. of *fuming* sulfuric acid (20 per cent free SO_3). Leaving the stopper off, heat the mixture on a wire gauze until the temperature

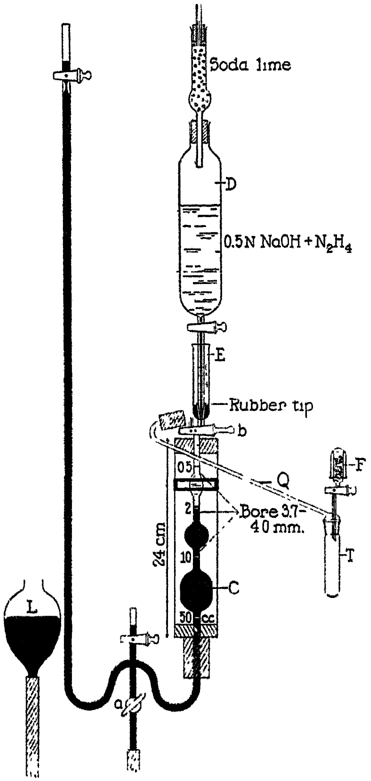

Fig. 1

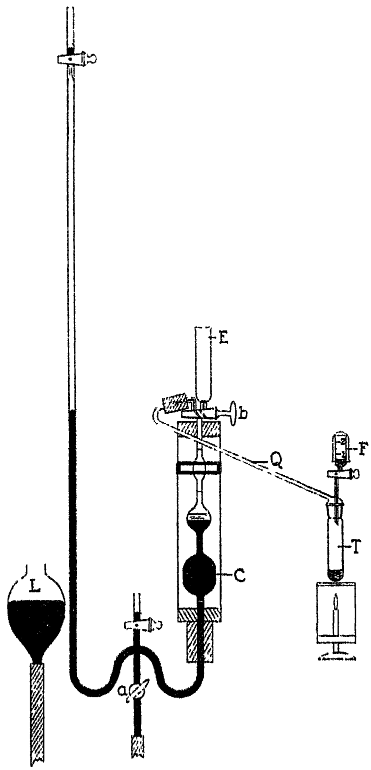

Fig. 2

Fig. 1. Apparatus after delivery of alkali into chamber, and before the start of the combustion.

Fig. 2. Apparatus at start of combustion. The chromic acid solution has been run into the combustion tube and the flame has just been brought under it, but, as shown by the low level of the mercury in the manometer, evolution of CO_2 and O_2 has not yet begun.

reaches 140–150°. Rotate the flask gently at times to assist solution of the chromic anhydride and escape of CO_2 formed by oxidation of any traces of organic matter that may be present.

When 150° has been reached, remove the flame, cover the open flask with an inverted, lipless, 150 cc. beaker, and let the mixture

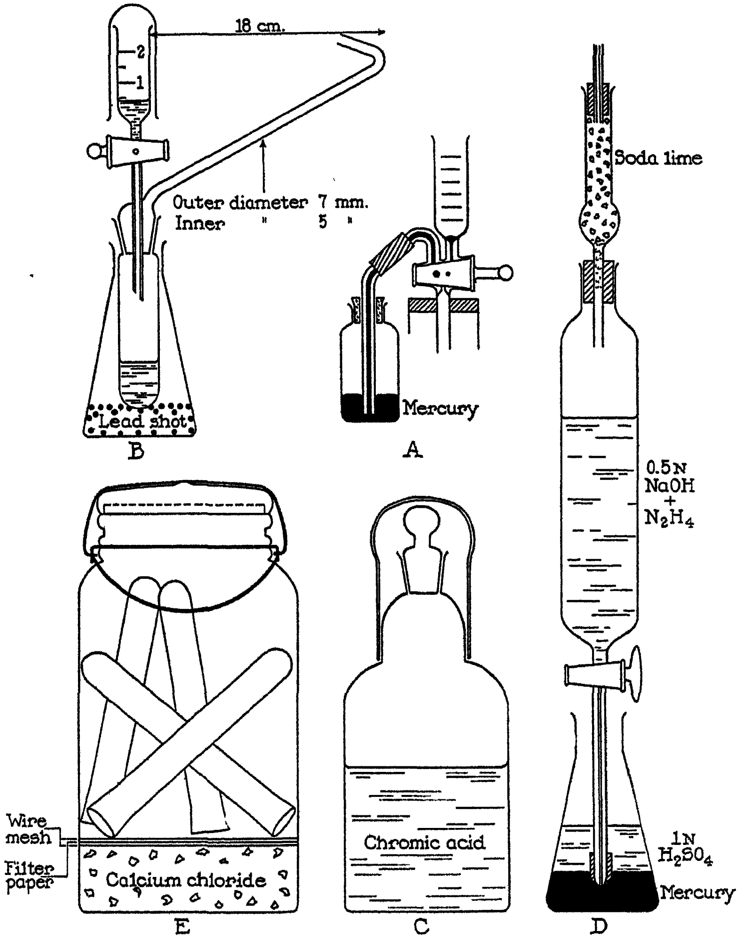

FIG. 3. Accessory apparatus. *A*, small bottle of mercury arranged for sealing capillary of cock *b* (Fig. 1); *B*, Pyrex 100 cc. flask weighted with small lead shot serving as a stand for combustion tube after analysis; *C*, 100 cc. bottle provided with cover and ground joint to prevent access of moisture to combustion fluid; *D*, cylinder for holding alkaline hydrazine solution, hung with tip protected from atmospheric CO_2; *E*, fruit jar arranged for drying and storing washed combustion tubes. 1 quart jars serve for the 15 cc. tubes used for microcombustions, and 2 quart jars for the 25 cc. tubes used in macrocombustions.

cool to room temperature. When cool, insert the glass stopper, but also keep the inverted beaker permanently over the stopper to prevent dust from settling on the rim of the flask and contaminating the fluid as the latter is poured out. For use in analyses a portion of the fluid is poured into a protected bottle, of 100 cc. capacity, as shown in Fig. 3, *C*.

The fluid must be protected from absorption of water from the atmosphere, as water diminishes the efficiency for combustion.

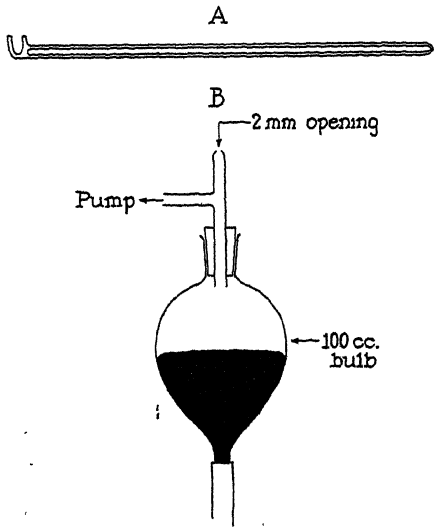

FIG. 4. *A*, glass tubes for measuring 100 and 200 mg. of pulverized KIO_3. The inside diameter is about 4 mm. The measuring receptacle can be made somewhat oversize, and ground down to exact measure on an emery wheel. *B*, attachment of suction pump to leveling bulb to raise and lower the mercury in chamber *C* (Fig. 1) without moving the leveling bulb.

Besides its oxidizing efficiency, the anhydrous acid mixture has the advantage of a high solvent power for chromic acid; it dissolves over 25 gm. of CrO_3 per 100 cc. at room temperature.

✓ Since the CrO_3 is hygroscopic and may vary in its moisture content, it is desirable to check the chromic acid concentration of the solution by titration. The chromic acid solution as made up, 5 gm. per 100 cc., would be nearly 0.5 M if the CrO_3 were anhydrous. The concentration shown by titration of our reagents has consistently been about 0.475 M. If a solu-

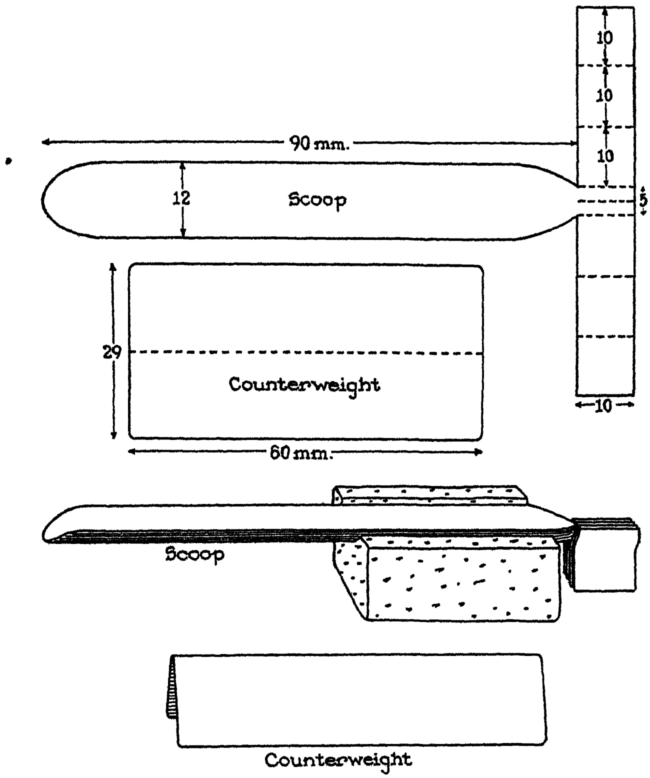

FIG. 5. Sheet aluminum scoop and counterweight used in weighing samples for microcombustions. The aluminum used is of 0.25 to 0.30 mm. thickness. The upper drawing indicates the pattern in which the sheet metal is cut, with the dimensions in mm., and the lines of sharp bends shown by broken lines. The scoop is shaped in a half cylinder by bending it about a rod of 7 mm. diameter. The back portion, with the folded metal, serves as a handle, and as a weight centered below the scoop to insure the latter against overturning on the balance pan.

tion as prepared is found to be weaker than 0.475 M, enough more CrO_3 should be added to bring it up to this concentration. The titration is carried out as follows:

Dilute a portion of the chromic acid solution to 10 volumes with water. Of the diluted solution measure 3 cc. into a 50 cc. Erlenmeyer flask, and add 5 cc. of water and 10 cc. of 10 per cent KI solution. After the flask has stood quietly for 5 minutes to complete the reaction between chromic acid and HI, the iodine produced is titrated with 0.1 N thiosulfate. (Cc.

of 0.1 N thiosulfate used × 0.1) = (molar concentration of chromic acid in the combustion fluid). This standardization should be repeated once a month, as the reagent loses strength on standing.

Potassium iodate, reagent grade, pulverized.

Approximately CO_2-free 0.5 N NaOH plus 0.3 M hydrazine. A solution of approximately CO_2-free NaOH of 0.815 ± 0.005 N concentration is first prepared sufficient to last for a season's combustions. A liter or two of distilled water is acidified with a few drops of dilute acid and is boiled to expel CO_2. The water is cooled in a flask protected from atmospheric CO_2 by a soda lime tube, and enough concentrated (18 to 20 N) NaOH solution, from which carbonate has been allowed to settle, is added to bring the solution to somewhat over 0.815 N concentration. A portion is titrated, and the remainder is diluted with a volume of water calculated to bring the concentration down to 0.815 N. The solution is stored in a paraffin-lined aspirator bottle, of which the stopper is provided with a soda lime tube, while the outlet connects with a capillary glass tube of 2 mm. bore and 12 or 15 cm. length; the rubber tube connecting the capillary tube to the bottle's outlet is of walls about 3 mm. thick.

From this solution the alkaline hydrazine solution is made in portions sufficient only to last for about a month, as the hydrazine slowly decomposes. Into a volumetric flask one weighs 2 gm. of hydrazine sulfate for each 100 cc. capacity of the flask. The capillary outlet of the 0.815 N NaOH bottle is washed free of any adhering carbonate, and a little of the alkali solution is wasted, to wash out any carbonate that may have formed in the outlet. The outlet capillary is then inserted nearly to the bottom of the flask with the hydrazine sulfate, and alkali is run in until the flask is about two-thirds filled. Without withdrawing the delivery tube, the flask is whirled a few times to dissolve the hydrazine. Alkali solution is then added until the flask is filled to the mark. The flask is stoppered and the solution mixed. The solution is then at once drawn by suction into the cylinder shown in Fig. 3, *D*. The last fifth of the alkaline hydrazine solution is left in the volumetric flask, as this portion is more likely to be contaminated with atmospheric CO_2.

When not being used for delivery of alkali, the cylinder with the alkali-hydrazine solution is kept with its tip protected from CO_2 as shown in Fig. 3, *D*.

The hydrazine in the alkaline solution loses about half its strength on standing for a month under ordinary laboratory conditions. It is adequate until the loss exceeds this amount. When alkali-hydrazine solution is made up, it should be dated, and at the end of a month any that is left is discarded.

The hydrazine content can be determined gasometrically in a few minutes as follows: The alkaline hydrazine solution is diluted 5-fold. 1 cc. of the dilute solution is measured with a stop-cock pipette into the chamber of the gas apparatus, and is followed by 1 cc. of saturated KIO_3 solution. The mercury in the chamber is lowered to the 50 cc. mark, and the chamber is shaken for 2 minutes to extract the N_2 formed by the reaction, $2NaIO_3 + 3N_2H_4 = 3N_2 + 2NaI + 6H_2O$. The p_1 reading is taken with the gas at 2 cc. volume. A blank analysis is made with water in place of the diluted hydrazine solution, and the manometer reading is taken as p_0. The pressure P_{N_2} of N_2 from hydrazine is calculated as $P_{N_2} = p_1 - p_0$. To calculate the molar concentration of hydrazine in the alkali reagent solution one multiplies P_{N_2} by 5 times the factor for a 1 cc. sample in Table 30 (fifth column) of Peters and Van Slyke (8), or in Table III (seventh column) of Van Slyke and Neill (12). (The fact that the S value stated in the tables is 3.5 cc., while it is 2 cc. in the hydrazine analyses, is without significant effect, because of the slight solubility of N_2.) The alkaline hydrazine solution as made up is 0.154 M in N_2H_4 concentration, and gives on analysis as described above a P_{N_2} of 280 mm. at 20°.

It is desirable to make the concentrations of NaOH and hydrazine sulfate exact, as they affect the solubility of CO_2 in the acidified reagent mixture from which the CO_2 is extracted at the end of the combustion, and the solubility affects the calculation factors.

2 N lactic acid. C.P. concentrated lactic acid (sp. gr. 1.20) is diluted with water to 5 volumes, and the concentration is checked by titration, with phenolphthalein as indicator.

Approximately 5 N sodium hydroxide. This is conveniently kept in a cylinder of the type shown in Fig. 3, *D*, but with no rubber ring about the delivery tip. The number of drops is determined in which 0.5 cc. is delivered.

Procedure

Placing Sample in Combustion Tube. (*a*) *Samples Weighed on Microbalance*—It is desirable to deposit the weighed sample in the bottom of the combustion tube, *T* (Fig. 1), without scattering any of the material on the upper walls where it might escape complete combustion. The aluminum scoop shown in Fig. 5

has been found convenient both for weighing and for depositing the sample in the combustion tube. Scoop and counterpoise are balanced within 0.1 mg., the scoop being kept slightly the heavier. The scoop and its counterpoise are kept in the balance case between weighings. They are then ready at temperature equilibrium with the balance, and the sample can be added and weighed without delay.

The counterpoise is placed on the right-hand pan of the balance, and a sample of dried substance sufficient to contain preferably between 2.5 and 3.5 mg. of carbon is placed in the scoop, while the latter, in the balance case, rests on its cork support as shown in Fig. 5. The scoop is lifted onto the left balance pan by means of the pincers with jaws lined with chamois skin. The scoop and sample are weighed at once. If the material is slightly hygroscopic, one can repeat the weighings at intervals of 1 minute for 3 minutes and obtain the correct weight by extrapolating back to zero time. Material too hygroscopic to be treated in this manner must be weighed in one of the customary closed tubes used in microanalyses of such material, but we have rarely found them necessary.

To transfer the sample to the combustion tube the latter is taken from its jar (Fig. 3, *E*) and is held horizontally with one hand while, with the chamois-protected pincers, the scoop with the sample is inserted into the tube. Tube and scoop are then tilted to a vertical position, depositing the sample in the bottom of the tube. If portions of the sample adhere to the scoop, the latter is struck sideways gently against the tube to dislodge the material. If a slight amount remains adherent, it does not matter, as the weighing is by difference.

The emptied scoop is returned to the balance pan, and the balance is closed and let stand 3 or more minutes for the scoop to regain temperature equilibrium. The empty scoop can be weighed at any time after 3 minutes. We prefer, however, to make the second weighing within at most 10 minutes, in order to forestall the possibility of changes in the zero point of the balance. If the second weighing is made within 10 minutes, and the temperature of the balance room is reasonably constant, we have found it as a rule unnecessary to recheck the zero point of the balance.

Because of its freedom from static electricity, its light weight, and the quickness with which it reaches temperature equilibrium,

the light aluminum foil has proved to be an ideal container for microweighings. We have on occasions finished a complete combustion, weighings included, within 20 minutes.

An alternative weighing procedure is to weigh the sample in a micro porcelain boat 15 to 17 mm. long, such as is used for microcombustions in furnaces. The boat with the sample is slid gently into the slightly inclined combustion tube, which is not turned vertically until the boat reaches the bottom. A sticky substance or non-volatile liquid can be handled in this manner.

The combustion tube with the sample is at once placed in a covered beaker, so that no opportunity is given for laboratory dust to fall into the tube.

(5) *Samples from Evaporated Solutions*—When submicrocombustions are carried out, with 0.3 to 0.7 mg. of carbon, the samples are usually too small to be weighed with 1/1000 accuracy on available microbalances, and must be measured into the combustion tubes as aliquots of solution, from which the solvent is then removed by evaporation. The concentration is so arranged that a sample of proper size will be contained in 1 or 2 cc. of solution. A 1 or 2 cc. pipette can be made to deliver an aqueous or alcoholic solution within 1 part per 1000. A weight burette, however, with a stem long enough to reach to the bottom of the combustion tube offers the most exact means of measuring the solution. It is desirable to use a volume of not more than 2 cc., in order not to have the solution run up the sides of the tube further than the combustion fluid to be added later.

To evaporate aqueous solutions in the combustion tubes the simplest and safest procedure is to leave the tubes overnight, somewhat inclined, in a large evacuated desiccator. The evaporation proceeds slowly, without danger of spattering, or of decomposition of heat-sensitive substances. Aqueous solutions of material which is not sensitive to heat can be evaporated by immersing the lower ends of the tubes in sulfuric acid in a Pyrex beaker which is heated on a steam bath, the top of the beaker being covered with a folded filter paper to keep out dust.

To remove volatile organic solvents, such as alcohol, ether, or acetone, the alundum pieces to prevent bumping are added (p. 511), and the tubes are placed in a beaker containing a 2 cm. layer of sulfuric acid. The top of the beaker is covered with a

filter paper and the beaker is heated on a steam bath until all visible solvent is evaporated. The warm beaker is removed from the bath and placed in a desiccator, which is evacuated three times, air filtered free from dust being admitted after each evacuation. When alcohol is the solvent, add 2 or 3 drops of water to the residue before the beaker is placed in the desiccator.

It is essential that the tubes be not left on the steam bath longer than is necessary to drive off the visible organic solvent. The dry residue is left in such a thin film that it is peculiarly accessible to oxidation by the air. Letting the tube heat for an hour after the film has dried may cause loss of 1 or 2 per cent of the carbon from a substance like cholesterol, which is ordinarily stable in air.

It is good practice to carry out the combustion within a few hours after the solvent has been evaporated. Even at room temperature some materials, when deposited in a thin film, appear to oxidize on standing, and yield low results when subsequently burned.

Connecting Combustion Tube with Manometric Chamber—Into the tube are dropped several pieces of the cleaned alundum or sand to insure smooth boiling, then 200 mg. of KIO_3 for a micro-, or 100 mg. for a submicroanalysis.[2] The KIO_3 may be measured by the tube in Fig. 4, *A*. With a medicine dropper a thick ring of syrupy phosphoric acid is then drawn around the upper part of the ground glass joint of *T* (Fig. 1), while *T* is in a horizontal position. Cup *F* of connecting tube *Q*, which has been resting as shown in Fig. 3, *B* in the combustion tube with which it was last used, is filled to the 2 cc. mark with fresh combustion fluid and the stopper is fitted to the combustion tube containing the sample to be burned. The combustion tube is now connected

[2] If a series of submicrocombustions is to be carried out, it may be convenient to prepare enough chromic acid solution with predissolved KIO_3 for the day's analyses. KIO_3 in the proportion of 50 mg. per cc. of the chromic acid solution is added to the latter, and is dissolved by heating not higher than 160°. The solution after cooling is transferred to the bottle in Fig. 3, *C*. At room temperature the solution is several times supersaturated with iodic acid, but the latter will remain dissolved for a day before significant amounts crystallize out.

with chamber C[3] as shown in Figs. 1 and 2, chamber C being completely filled with mercury.

Preliminary Ejection of Air—Cock b (Fig. 1) is turned to connect C and T, and the mercury in C is lowered to the 50 cc. mark. This procedure draws about two-thirds of the air from Q and T over into C. Cock b is then closed, mercury is readmitted into C, the air trapped over the mercury in C is ejected through cup E, and cock b is closed again.

The ejection of the air from the system has two advantages. One is that removal of the air diminishes to insignificance the part of the blank that is due to atmospheric CO_2 present in the apparatus when the combustion is begun.

A second advantage is that when the CO_2 formed by combustion is transferred from the combustion tube to the alkali solution in C (Fig. 1), by running the gases back and forth between C and T as described later, the absence of the inert gases of the ejected air halves the number of excursions required for the transfer. The fewer the interfering molecules of inert gas the quicker the path of the CO_2 molecules to the absorbing solution; hence speed of CO_2 absorption by the alkali solution varies inversely as the proportion of inert gases mixed with the CO_2.

Measurement of Alkaline Hydrazine Solution into Manometric Chamber—2 cc. of the alkali-hydrazine solution are measured from D into C through a mercury seal, as shown in Fig. 1. (The solution should be at the same room temperature as the water in the jacket of C.) The admission of the alkali is best regulated by opening cock b wide and controlling the flow of mercury through cock a. The admission is stopped when the mercury in C falls to a level about 1 mm. above the 2 cc. mark, as shown in Fig. 1. Then D is withdrawn and mercury from E is let into the chamber to fill the capillaries of cock b. The displacement of the slight volume of solution from the capillary of b into the chamber brings the volume of solution in C exactly to the 2 cc. mark. Cup E is rinsed with

[3] For connecting tube Q to the capillary of C we use a thick tube of soft, elastic rubber; to fit over 7 mm. glass tubes the bore is 5 to 5.5 mm. and the walls are 5 to 6 mm. thick. With this the continual connecting and disconnecting are easier than with thin rubber, and the connection is more secure.

acidified water; this cup is never permitted to stand with its walls wet with alkali.

Combustion—*L* is lowered, and mercury is drawn out of *C* and the manometer until the mercury in the manometer is about level with the 2 cc. mark on *C*. Cock *a* is then closed, and cock *b* is turned to connect chamber *C* with the combustion tube. The leveling bulb is then placed, as shown in Figs. 1 and 2, at such a height that the mercury surface in it is about level with the 50 cc. mark on *C* and remains at this level until the combustion is finished. A measured volume of combustion fluid is now run from *F* into *T*; for microcombustions, with 2 to 3.5 mg. of carbon, 2 cc. of the fluid are used, while for the submicroanalyses, with 0.3 to 0.7 mg. of carbon, only 1.5 cc. are taken. The micro flame is now brought under the combustion tube, and the arrangement is as shown in Fig. 2.

Fine bubbles of CO_2 gas begin to rise in the combustion fluid as soon as the latter is warmed. A minute or two is taken to warm the fluid to boiling. Preferably the CO_2 evolution should not be so rapid that it makes at any time a foam collar more than 2 cm. high on the fluid; although even if it should fill the greater part of the tube the analysis is not necessarily lost. After the initial CO_2 evolution the fluid is heated rapidly to boiling.

As CO_2 and O_2 are evolved, the mercury falls in *C* (Fig. 1) and rises in the manometer. Cock *a* is slightly opened every few seconds at this stage to admit mercury from *L* into *C* and keep the gas space in *C* at about 1 cc. Within about a minute from the beginning of heating enough gas has been evolved to press the mercury in the manometer up to its top, and to permit complete opening of cock *a* without causing backflow of alkali solution from *C* to *T*. Cock *a* is now left fully open during the rest of the combustion, and the boiling proceeds quietly at about 150 mm. less than atmospheric pressure. Vigorous boiling at about 600 mm. pressure is continued, with foam filling one-third to one-half of the tube, for 1.5 minutes to complete the combustion. Of the substances analyzed we have not found any which required more than 1 minute's vigorous boiling for complete combustion. Even the fatty acids, usually among the more resistant substances in combustion, were burned completely in 1 minute, after the pressure

had reached 600 mm. With many substances combustion is complete as soon as this point is reached.

Making the boiling vigorous is emphasized because it has been found essential with palmitic and stearic acids. With most substances gentle boiling suffices. The higher fatty acids, however, as the combustion fluid is warmed, melt and form a film on the surface. It is perhaps because of this behavior that vigorous boiling is essential with them. Gentle boiling (with a foam collar under 5 mm. high) may yield results 0.5 to 1 per cent too low with palmitic and stearic acids.

It is unnecessary and undesirable to continue the boiling longer than 1.5 minutes. By the end of this time the chromic acid has evolved practically all of its labile oxygen, and the iodic acid begins to decompose and give off iodine and oxygen. If heating were continued until all the iodic acid had been decomposed, the volume of additional O_2 evolved would retard appreciably the absorption of CO_2 next described, so that 25 instead of 20 excursions of the mercury in *C* (Fig. 1) might be required for complete absorption. If the period of boiling at 600 mm. pressure is kept under 2 minutes, so little iodic acid is decomposed that the iodine vapors are barely visible in the combustion tube.

In case any particles of the sample have been dropped on the upper parts of the combustion tube, or in case the foaming during the initial CO_2 evolution has been so rapid that the foam reached above the half-way mark of the tube, it is a desirable precaution, after boiling at 600 mm. has been established, to shake the combustion tube so that the oxidizing liquid will reach any unburned material in the upper part of the tube. Or the flame may be increased for a few seconds so that the boiling fluid reaches the upper part of the tube.

The volume of O_2 evolved from the reagents can be roughly estimated as follows: After the CO_2 has been absorbed, but while the combustion tube is still connected and the flame is still under the combustion tube, one sets the mercury meniscus in the chamber at the 50 cc. mark, closes cock *a* (Fig. 1) for a moment, and notes the height of the mercury in the manometer. If it does not lie more than 350 mm. above the mercury surface in *C*, the amount of O_2 present is not enough to prevent complete absorption of the CO_2 by 20 excursions of the mercury (see the next paragraph). If by unduly long boiling of the reagents, or leakage of air into

the apparatus, the non-CO_2 gases exert a pressure greater than 350 mm., it is well to use a few more excursions to make certain of 100 per cent absorption.

Absorption of CO_2 by Alkali in Gas Chamber—After the combustion is completed, the flame is left under the tube exactly as during the combustion, while the mercury in *C* (Fig. 1) is lowered and raised 20 times to cause complete transfer of CO_2 to the alkali solution in *C*. The 20 excursions should take about 3 minutes. At each lowering of the leveling bulb the mercury in *C* is dropped to about the 50 cc. mark, and the fluid in *T* boils vigorously. The tube should then fill with foam. At each raising the bulb is lifted till the gas space in *C* is compressed to about 5 cc. After 5, 10, 15, and 20 excursions of the mercury in this manner, the respective percentages of the CO_2 transferred to the alkali solution in *C* have been found to be 91, 98.3, 99.7, and 100 respectively.

When many combustions are carried out, it will be found convenient to avoid the labor of the many raisings of the mercury bulb by alternately applying and releasing suction at the top of the bulb, without moving the bulb from its usual level shown in Fig. 2. A good water aspirator will raise and lower the mercury at about the same rate as lifting the bulb by hand. A simple device for releasing the suction is shown in Fig. 4, *B*. With the pump connected directly, without an intermediate safety flask, suction is obtained by merely closing the open tube by pressure of any flat rubber surface, and is released by removing the closure. Or a 3-way cock may be substituted; this is desirable if the suction tube is connected to an evacuated reservoir, in which it is preferable not to release the vacuum when the latter is released in the leveling bulb. When the suction method is used, it is desirable to have a mercury bulb of not over 100 cc. capacity, and to have sufficient mercury in the system so that the bulb will be nearly filled when all the mercury is withdrawn from *C* (Fig. 1). If there is a large gas space in *L*, it is more difficult to control the excursions of the mercury in *C*.

After absorption is completed, the flame is removed from the combustion tube, cock *b* (Fig. 1) is closed, and tube *Q* is disconnected from the chamber. The hot combustion tube is taken with tongs or a strong metal test-tube holder, and stood in a flask as shown in Fig. 3, *B*.

Ejection of Unabsorbed Gases—Before O_2 and N_2 are ejected, the curved inlet capillary above cock *b* is filled with mercury drawn in from a small bottle as shown in Fig. 3, *A*. About as much mercury is left in this capillary above the cock as is shown in Fig. 1.

Then, with cock *b* closed, the gases in *C* are put under positive pressure by raising leveling bulb *L* a little above cock *b*. With the bulb at this level, cock *a* is closed and *b* opened to connect chamber *C* with cup *E* above it. Mercury is then admitted from *L* into chamber *C* until the rising alkali solution, driving the gases out through *E*, just reaches the bottom of cock *b*. In succession one then closes cocks *a* and *b*. The leveling bulb is lowered to the position shown in Figs. 1 and 2, and a little mercury is admitted from cup *E* into the chamber to seal the connecting capillary. A small bubble of air, trapped in the capillary, is thus readmitted into *C*, but it has no influence on the CO_2 determination, since it is CO_2-free.

Extraction of CO_2 and Reading of p_1—Exactly 1 cc. of 2 N lactic acid is measured into chamber *C* from an accurate stop-cock pipette provided with a rubber-ringed tip. The admission is made through a mercury seal in the same manner shown in Fig. 1 for admission of alkali. Mercury to fill the connecting capillary between *E* and *C* is admitted after the acid.

The mercury in the chamber is then lowered to the 50 cc. mark, cock *a* leading to the leveling bulb is closed, and the chamber is shaken 20 or 30 seconds, extracting most of the CO_2 from the solution. The CO_2 that has entered the gas phase increases the pressure there enough to force the mercury down below the 50 cc. mark. To correct this displacement, enough mercury is admitted from the leveling bulb to bring the top of the mercury meniscus in the chamber exactly to the 50 cc. mark. The chamber is then shaken for 1.5 minutes to complete extraction of CO_2 from the solution.

Mercury is now admitted from the leveling bulb until the volume of the gas phase in the chamber is reduced, to 10 cc. for the microcombustions with 2 to 3.5 mg. of carbon, or to 2 cc. for the submicrocombustions with a fifth as much carbon. In admitting the mercury the precautions described by Van Slyke and Neill ((12) p. 533) or by Peters and Van Slyke ((8) p. 277) are observed, to prevent undue reabsorption of CO_2. The chief precautions are to complete the admission of the mercury within a space of 30 or 40 seconds, and to avoid setting the mercury in the chamber and manometer to oscillating by jerky opening or closing of cock *a*. If these precautions are followed, the reabsorption

of CO_2 that occurs during the admission of the mercury is so constant (estimated as 0.7 per cent when the gas is compressed to 10 cc., 1.6 when it is compressed to 2 cc.) that variations in reabsorption affect results by not more than 1 part per 300 when the gas is brought to 2 cc., or 1 part in 1000 when it is brought to 10 cc. As the meniscus of the solution in the chamber approaches the 2 or 10 cc. mark it is desirable to watch it with a hand lens, in order to stop it exactly on the line.

The reading, p_1, is then taken on the manometer. If it is desired to check this reading, the mercury in the chamber is lowered again to the 50 cc. mark, shaken for a minute, and the gas is returned to 2 or 10 cc. volume for repetition of the p_1 reading. With practice it will be found that the duplicate readings check so closely that the repetition is unnecessary.

Reabsorption of CO_2 and Reading of p_2—After the p_1 reading has been taken, the cock leading to the leveling bulb is opened, while the bulb is left at the level shown in Figs. 1 and 2, so that the gas in the chamber is under slight negative pressure. Into cup *E* one measures 0.5 cc. of the 5 N sodium hydroxide solution; the measurement is most conveniently made by counting the drops. The alkali is then admitted into the chamber, with care not to admit any air into either the chamber or the capillary above cock *b*. In the submicrocombustions, with less than 2 cc. of CO_2 present, absorption of all the CO_2 occurs about as rapidly as the alkali can be run in. When the larger amounts of CO_2 measured at 10 cc. volume are present, however, it takes a little longer to complete the absorption, and one admits the alkali over a period of a half minute or more. When all the alkali except enough to fill the capillary below the cup is in the chamber, one pours 2 or 3 cc. of acidified water into the cup, followed by about 0.5 cc. of mercury. The mercury is then run into the chamber, dislodging any of the alkali solution which may have stuck in the part of the chamber under cock *b*.

Sometimes the somewhat viscous 5 N alkali solution flows in a solid column into the 4 mm. tube at the top of the chamber, instead of streaming down the walls and absorbing the CO_2. In this case one follows the alkali with about 1 cc. of mercury, which is admitted in several fine jets. These dislodge the alkali and cause quick absorption of the CO_2.

To mix the solutions in the chamber, and to insure absorption of the last traces of CO_2, one now lowers and raises the mercury in the chamber three times, each lowering bringing the surface of the solution in the chamber, not to its bottom, but only to a point a little below the 10 cc. mark, and each raising bringing the pressure up to about atmospheric.

The solution meniscus in the chamber is then brought to a point a little below the 2 or 10 cc. mark at which p_1 was read, and is let stand there for 1 minute while the solution drains down from the walls above the mark. During this minute one can read the temperature in the water jacket of the chamber and look up the corresponding carbon factor. Then the meniscus is raised exactly to the 2 or 10 cc. mark and reading p_2 is made on the manometer. The pressure, P_{CO_2}, of CO_2 from the combustion is calculated as $P_{CO_2} = p_1 - p_2 - c$, where c is the value of $p_1 - p_2$ obtained in a blank analysis.

Washing Chamber after Analysis—To clean the chamber for the next analysis the used alkaline lactate solution is ejected and the chamber is washed once with dilute acid and once with water. A rapid and convenient technique is the following. The mercury leveling bulb is lowered about 80 cm. below chamber *C* (Fig. 1) so that the mercury will all drain out of the chamber. While it is draining, a few drops of the 2 N lactic acid are placed in the cup at the top of the chamber, together with enough water to fill the cup. The acidified water (but no air) is now let into the evacuated chamber. The leveling bulb is then raised and the solution expelled from the chamber. The washing is then repeated in the same manner, except that only distilled water is used. After the water is ejected, the mercury is lowered once more to the bottom of the chamber, and is allowed to rise rather slowly, so that the film of water adherent on the sides of the chamber is detached and floats up on the mercury. The drop of water thus collected, together with about 1 cc. of mercury, is run up into the cup, and the apparatus is ready for the next combustion.

Alternative Rapid Analysis without Reabsorption of CO_2—This procedure is slightly less exact, but saves 3 or 4 minutes on each analysis, and serves well when series of analyses are required, as in routine blood fat determinations, in which an increase of a mm. in the error of P_{CO_2} is not important.

The analysis is carried out, as described above, until the p_1 reading has been taken, and this reading finishes the analysis. P_{CO_2} is calculated as $P_{CO_2} = p_1 - p_0$, where p_0 is the p_1 reading obtained in a blank analysis at the same temperature. If the blank analysis is performed within 3° of the same temperature as the unknown, the blank reading can be corrected to apply to the unknown by calculating p_0 as (p_1 of the blank) + (rise in p_{H_2O}), where the "rise in p_{H_2O}" is the increase in vapor tension of water caused by the temperature change from the temperature of the blank analysis to the temperature of the substance analysis; *e.g.*, if the blank is run at 20°, where the vapor tension of water is 17.4 mm., and the carbon analysis is run at 22°, where the vapor tension is 19.6 mm., the p_0 used is the p_1 of the blank plus 2.2 mm. If the temperatures were reversed, the blank being at 22° and the unknown at 20°, the p_0 used would be the p_1 of the blank minus 2.2 mm., the "rise in p_{H_2O}" being negative. For convenience in correcting p_0 values for temperature changes the vapor tensions of water are given in the last column of Table I.

Between analyses performed without reabsorption of CO_2 it is necessary to wash chamber *C* (Fig. 1) only once, with water, since the amount of CO_2 in the film of acid solution left after the reagents have been ejected from the chamber is slight.

Calculation of Results of Micro- and Submicroanalyses

The mg. of carbon in the sample are calculated by the factors of Table I, corrected if necessary by a factor obtained from control analyses of a standard pure substance, as described later.

$$\text{Mg. carbon} = P_{CO_2} \times \text{factor}$$

Derivation of Factors for Micro and Submicro Carbon Calculation

In Equation 3 of Van Slyke and Sendroy (14) the formula is given for calculating mM of CO_2 from the pressure exerted by the gas in the manometric determination. Multiplying this formula by 12.01, the atomic weight of carbon, gives as the formula for the factor, by which P_{CO_2} is multiplied to give mg. of carbon,

$$(1) \qquad \text{Factor} = \frac{0.0007099ia}{1 + 0.00384t}\left(1 + \frac{S}{A - S}\,\alpha'\right)$$

The symbols have the following significances: a is the volume of the CO_2 gas when its pressure is measured; i is an empirical correction for a small

TABLE I

Factors for Carbon Calculation

Temperature	Factors			Vapor tension of water
	Submicro-analysis $a = 2.000$ $S = 3.00$ $i = 1.016$	Microanalysis $a = 10.00$ $S = 3.00$ $i = 1.007$	Macroanalysis $a = 46.00$ $S = 4.00$ $i = 1.000$	
°C.				mm.
10	0.001474	0.007303	0.03320	9.1
11	66	265	304	9.8
12	58	228	289	10.4
13	51	192	274	11.1
14	44	157	269	11.9
15	37	122	244	12.7
16	30	088	229	13.5
17	24	054	215	14.4
18	17	020	201	15.3
19	10	0.006987	187	16.3
20	03	954	173	17.4
21	0.001397	922	159	18.5
22	90	890	145	19.6
23	84	859	132	20.9
24	78	828	119	22.2
25	72	798	106	23.5
26	66	769	093	25.0
27	60	740	080	26.5
28	54	711	067	28.1
29	49	683	055	29.7
30	43	655	043	31.5
31	37	628	031	33.4
32	32	601	019	35.3
33	27	575	007	37.4
34	21	549	0.02996	39.5
35	16	523	985	41.8

When determining total plasma lipids by combustion, following Van Slyke, Page, and Kirk (13), one may multiply the carbon factors by 1.266 in order to obtain factors for calculation of mg. of total lipids directly from P_{CO_2}.

When cholesterol is determined by combustion of the digitonide, $C_{27}H_{46}O \cdot C_{55}H_{94}O_{28}$, one may multiply the factors of Table I by 0.3926, in order to obtain a table of factors for calculating mg. of cholesterol from P_{CO_2}.

amount of CO_2 reabsorbed when the gas volume is diminished from $A - S$ to a cc.; t is temperature; S is the volume of acidified solution from which the CO_2 is extracted; A is the total volume of the extraction chamber measured at the bottom mark ($A = 50$ cc. in Fig. 1); α' is the distribution coefficient of CO_2 between the gas phase and the liquid phase in the chamber ($\alpha' = CO_2$ per cc. of liquid $\div$ CO_2 per cc. of gas).

In the analyses described above, a has the value 2 cc. for the submicro-, 10 for the microanalysis. The factor i has been determined by combustion analysis of pure crystalline glucose, as Van Slyke and Sendroy determined it by analysis of Na_2CO_3. The value of i from glucose combustion has been found to be 1.016 when a is 2 cc., agreeing closely with the value 1.017 found by Van Slyke and Sendroy (14) in analysis of standard carbonate. When a is 10 cc., and the gas is compressed only to this volume for pressure measurement, reabsorption is less, and the i value has been found to be 1.007, indicating only 0.7 per cent reabsorption. The value of A in the present analyses is 50.00 cc., and of S is 3.00 cc.; the ratio $S/(A - S)$ is 0.638.

The value of α' in the acidified lactate solution has been determined at temperatures from 17–32° by the gas solubility method of Van Slyke (11). The solubility of CO_2 in the lactate solution was found at 17° to be 78.4 per cent of the solubility in water; at 25°, 79.9 per cent; and at 32°, 81.3 per cent. Over this temperature range the value of α' for the lactate may accordingly be calculated from the α' of water by the formula

$$\alpha'_{\text{lactate}} = \alpha'_{H_2O}\ (0.752 + 0.0019t) \tag{2}$$

With the above values for the constants, the factors for carbon are calculated by the following formulae.

For the submicroanalysis, with $a = 2$ and $i = 1.017$, we have

$$\text{Factor}_{(a=2)} = \frac{0.0014425}{1 + 0.00384t}\ (1 + 0.0638\ [0.752 + 0.0019t]\ \alpha'_{H_2O}) \tag{3}$$

where the α'_{H_2O} values are those given in Table I of Van Slyke and Neill (12), and in Table 27 of Peters and Van Slyke (8), as calculated from Bohr and Bock's original data.

Similarly, for the microanalysis, with $a = 10$ and $i = 1.007$ we have

$$\text{Factor}_{(a=10)} = \frac{0.007149}{1 + 0.00384t}\ (1 + 0.0638\ [0.752 + 0.0019t]\ \alpha'_{H_2O}) \tag{4}$$

Correction of Factors by Combustion of Pure Substance

In the theoretically calculated factors, several constants are involved which may vary significantly with different sets of apparatus. The volumes, a and A, of the chamber are such constants. The correction of the thermometer in the water jacket of the chamber is another. When a microbalance is used to weigh

the samples, the accuracy, in absolute units, of the rider on the balance is another determining constant, and one which cannot be checked within 1 part per 2000 unless a correspondingly exact standard 10 mg. weight is available. Each of these constants can be measured by itself, but, particularly when the rider correction for a microbalance is included, the simplest method of correcting for deviations in all of these constants is to perform a series of careful analyses on an easily analyzed substance of high purity, and include all the corrections in one factor which is calculated from the mean result. Calling this factor b, one calculates it as

$$(5) \qquad b = \frac{\text{carbon present}}{\text{carbon calculated from } P_{CO_2} \text{ by factor in Table I}}$$

Both micro- and submicrocombustions with known samples are performed, and a b value is determined for each of the two types of combustion. Then an individual table of carbon factors for the apparatus used is prepared by multiplying by these values of b the factors for the micro- and submicroanalyses, respectively, in Table I.

An example of a determination of the b factor is given in Table II.

With any apparatus in which the 2 and 10 cc. marks are as accurate as in most chambers furnished by the better manufacturers, the factors in Table I can be used unchanged for many routine purposes. For combustions of assured accuracy, however, it is desirable to control all constants together by determining the b value and with it preparing one's own table of factors by correction of those in Table I.

The most convenient way to use the factors is to plot them against temperature on a sufficiently large scale to permit reading with an accuracy of 1 part in 3000.

Analyses at Low Room Temperatures—The analyses by which the method has been controlled have all been made at temperatures above 18°, and most of them at 22° or higher. At lower temperatures it is possible that both the absorption of CO_2 by alkali in C (Fig. 1) after the combustion, and the subsequent extraction of the CO_2 after acidification, may take more time than has been allotted in the above descriptions. It may be well, therefore, for analyses made below 18°, to increase the number of

excursions of the mercury for absorption of CO_2 by alkali in *C* from the prescribed 20 to 25, and to prolong the extraction period from 1.5 minutes to 2 minutes, or to 2.5 for temperatures below 15°. The factors for temperatures between 10–17° are calculated from extrapolated values of α', and may be in slight error from the extrapolation, but the error probably does not exceed 1 per 1000.

Economy of Time during Analysis—When one is analyzing samples of organic substances weighed on a microbalance, one can utilize the extraction period for the weighing of the aluminum scoop with the sample for the next combustion. If the weighing takes more than 1.5 minutes, it does not matter, as the shaking out of the CO_2 in the gas chamber may be prolonged without affecting the results. After the weighing the sample is deposited in a combustion tube for the next analysis, and the scoop is returned to the balance pan, where it reaches temperature equilibrium while the current analysis is being finished. The weighing of the empty scoop is then carried out.

When one is analyzing material in a series of tubes with residues from solutions (*e.g.*, plasma lipids), one can use each boiling period for calculation of results of the preceding analysis, and utilize the extraction period for preparation of the combustion tube for the next analysis.

Care of Apparatus When Not in Use—When a day's combustions are finished, cup *F* (Fig. 1) is washed out with syrupy phosphoric acid, and the cock is removed, lubricated freely with the phosphoric acid, and replaced. If this is not done, iodic or chromic acid may crystallize about the cock with time and absorption of moisture, and freeze the cock. If the cock does freeze from neglect of the precaution, it can usually be loosened by hot water and manipulation; but treating it with H_3PO_4 at the end of the day will entirely prevent the trouble. The connecting tube *Q* is left inserted in the combustion tube last used, which is stood in a weighted Erlenmeyer flask as shown in Fig. 3, *B*. A cover is placed over the flask and tube to keep away dust.[4]

[4] A convenient cover is the light, transparent Cello-Metal shield rectangular form, made of cellophane and aluminum, provided by the Fisher Scientific Company of Pittsburgh. The size used is with base 8 $\times$ 14 inches and height of wall 12 inches.

The combustion tubes used in a series of analyses are collected in a beaker, which is kept covered with an inverted Petri dish to prevent access of dust. When the series is completed, the tubes are rinsed out three times with clear tap water and three times with distilled water. They are then placed in a covered vessel where they can drain and dry. As such a vessel we have found a 1 quart fruit jar arranged as shown in Fig. 3, *E* to be satisfactory. Tubes placed in it are dry for use again within an hour or two, and they are so completely protected from dust that they may be left for months without increasing the values they yield in blank analyses. The *tubes are never left open in the circulating air of the laboratory*, except for the moments when they are receiving samples for analysis.

If there is any reason to suspect that a combustion tube has become contaminated with dust or organic matter, it is laid aside, and is not used again until it has been cleaned by heating it in chromic acid cleaning mixture to 120–140°, then rinsed in distilled water, and dried as in Fig. 3, *E*.

Before new combustion tubes are used they are always cleaned in this way. Merely soaking in cold chromic-sulfuric acid mixture may not suffice.

If the connecting tube *Q* (Fig. 1) should become contaminated, it is cleaned by removing the stopper and laying tube and stopper in a large evaporating dish with hot chromic-sulfuric acid. Tube and stopper are then washed and dried in an oven or desiccator.

If at any time alkali from the chamber gets into the connecting tube (*Q*, Fig. 1), the analysis is discarded; the tube is rinsed out with dilute hydrochloric acid, and is dried before being used again.

EXPERIMENTAL. MICRO- AND SUBMICROCOMBUSTIONS

Determination of Correction Factor of Apparatus for Microcombustions—Several analyses of Bureau of Standards anhydrous glucose were performed to determine the *b* correction factor of the apparatus. The weighings were made on a microbalance the rider of which had been checked against a standard 10 mg. weight which was believed to be exact to within 2 γ.[5] The *b* correction factor calculated from the mean of the glucose analyses is 1.0007

[5] For the loan of this standard weight we are indebted to Dr. Theodore Shedlovsky, who had calibrated it.

for the apparatus used. The difference from unity is hardly significant, but we have multiplied the micro carbon factors of Table I by 1.0007 for use in the analyses of Table III with this apparatus.

Microcombustions of Various Types of Substances—In Table III are given results with a number of substances, aromatic, aliphatic, containing nitrogen in various forms, chlorine, and sulfur. For the methionine and cystine we are indebted to Dr. Vincent du

TABLE II

Microanalysis of Crystalline Glucose, with Determination of Correction Factor of Apparatus Used

Calibration of chamber marks, a = 10.008 cc., A = 49.95 cc. Theoretical carbon content of glucose, 40.00 per cent.

Glucose sample b	p_{CO_2} c	Temperature d	Carbon factor from Table I, uncorrected e	Carbon content of sample calculated by uncorrected factor $= \frac{100\ ce}{b}$
mg.	*mm.*	*°C.*		*per cent*
6.011	349.8	23.0	0.006859	39.91
6.954	405.2	23.4	847	39.89
7.928	463.0	24.0	828	39.88
7.828	456.0	23.0	858	39.95
7.402	432.8	23.5	844	40.02
6.894	403.8	23.9	831	40.01
8.229	484.2	24.9	801	39.92
8.132	478.3	24.5	813	40.07
7.818	462.1	25.6	787	40.11
Mean				39.97

Correction factor found = 40.00/39.97 = 1.0007.

Vigneaud. The lysine picrate was prepared from gelatin and recrystallized three times. The arginine monochloride was also from gelatin, the arginine being precipitated as flavianate, then recrystallized by Dr. Robert Dillon as the monobenzoyl compound, and finally converted into the monochloride and crystallized from alcohol. The cholesterol was a commercial preparation which had been recrystallized. The palmitic acid had been recrystallized three times from alcohol. The stearic acid, tryptophane, and tyrosine were Eastman Kodak preparations. The

TABLE III

Microcombustions

Substance	Sample	Duration of boiling at about 600 mm.	P_{CO_2} at 10.008 cc.	Temperature	Factor*	Carbon		
						Found		Theoretical
	mg.	*min.*	*mm.*	*°C.*		*mg.*	*per cent*	*per cent*
Lysine monopicrate	7.461	1	420.7	24.8	0.006808	2.864	38.38	38.40
	6.378	2	358.8	24.5	818	2.446	38.35	
	7.833	3	440.7	24.5	818	3.005	38.36	
" dihydrochloride	8.464	2	407.7	23.4	852	2.793	32.98	32.88
	8.015	3	388.0	23.8	840	2.654	33.11	
	8.161	3	391.8	24.1	830	2.676	32.79	
Arginine monochloride	7.084	0	356.9	24.0	833	2.439	34.43	34.21
	5.993	1	302.8	24.5	818	2.064	34.45	
	7.186	2	363.3	25.0	803	2.472	34.39	
Cystine	7.341	2	324.7	25.4	791	2.205	30.04	29.99
	6.881	3	303.2	25.6	785	2.057	29.90	
	8.417	3	369.6	24.1	830	2.524	29.99	
Methionine	9.171	2	549.2	26.1	771	3.717	40.54	40.25
	7.951	3	470.1	23.8	839	3.215	40.44	
	7.191	3	425.4	24.0	833	2.906	40.42	
Tryptophane	5.194	1	497.8	27.5	730	3.340	64.52	64.69
	5.458	2	523.8	27.9	719	3.520	64.48	
Tyrosine	3.901	0	346.3	28.1	713	2.325	59.59	59.66
	4.612	1	409.5	28.5	702	2.744	59.51	
	4.748	2	412.6	23.2	858	2.830	59.60	
Cholesterol	3.539	1	435.0	24.0	832	2.972	83.98	83.87
	3.236	1	397.7	24.3	824	2.714	83.87	
	3.474	2	425.4	23.1	861	2.919	84.02	
	3.663	2	456.1	27.2	739	3.074	83.91	
	3.370	3	412.8	23.4	852	2.829	83.93	
	3.769	3	467.0	25.3	794	3.173	83.99	
Stearic acid	4.131	1	466.8	27.7	724	3.139	75.98	76.00
	4.461	1	503.2	27.8	721	3.382	75.80	
	4.132	2	464.0	26.6	756	3.134	75.86	
	3.975	2	447.9	26.6	756	3.026	76.13	
	4.218	3	473.8	26.7	754	3.200	75.87	
Palmitic "	4.340	1	474.2	23.3	855	3.251	74.90	74.94
	3.995	2	438.5	24.0	833	2.996	75.00	
Benzoic "	4.269	2	430.3	22.9	867	2.954	69.22	68.85
	4.921	3	494.7	23.6	845	3.386	68.81	

TABLE III—*Concluded*

Substance	Sample	Duration of boiling at about 600 mm.	P_{CO_2} at 10.008 cc.	Temperature	Factor*	Carbon		
						Found		Theoretical
	mg.	*min.*	*mm.*	*°C.*		*mg.*	*per cent*	*per cent*
Silver acetate	19.050	1	401.8	24.9	0.006806	2.733	14.35	14.37
	18.703	2	393.6	23.9	836	2.691	14.39	
	18.227	3	384.6	24.2	827	2.624	14.40	
Pentaacetyl glucose, $C_{16}H_{22}O_{11}$	4.983	1	359.3	24.1	830	2.454	49.25	49.23
	4.834	2	351.4	24.6	813	2.394	49.53	
	4.885	3	354.3	25.0	803	2.409	49.33	
Nitrobenzylheptaacetyl gentiobioside, $C_{33}H_{41}O_{20}N$	4.402	1	330.6	24.0	833	2.259	51.33	51.34
	5.531	2	416.0	23.8	840	2.845	51.44	

For some of the analyses in this table we are indebted to Dr. R. M. Archibald.

* The factors are those of Table I multiplied by 1.0007 (see Table II).

silver acetate was checked by Dr. Alma Hiller by sulfocyanate titration of its silver, which was found to be 64.67 per cent, calculated 64.64. For the pentaacetyl glucose and nitrobenzylheptaacetyl gentiobioside we are indebted to Dr. Walther Goebel. The gentiobioside had been six times recrystallized. All the samples were weighed on a microbalance with the aluminum scoop and counterweight in the manner previously described. It will be noted that 2 minutes boiling was required for complete combustion of the cholesterol, whereas the other materials that were tried with shorter periods were burned in 1 minute, measured from the time the pressure reached 600 mm. The recorded durations of boiling do not include all the time that the material was heated with the combustion fluid; about 1 minute is required to start the boiling, and during this time the temperature is rising from room temperature to boiling. During this period of about a minute the greater part of the combustion occurs; in fact it is probably complete by the time boiling at 600 mm. starts with the less resistant materials, such as glucose and most amino acids. The chromic acid is nearly all decomposed by the heat, by the time the first measured minute of boiling at about 600 mm. is over, and any residues burned later are presumably oxidized by the iodic acid, which is more resistant to heat decomposition.

Submicrocombustions—Examples of the results with these are given in Table IV.

TABLE IV
Submicrocombustions

Substance	Sample	P_{CO_2} at 2.008 cc.	Temperature	Factor*	Carbon		
					Found		Theoretical
	mg.	*mm.*	*°C.*		*mg.*	*per cent*	*per cent*
Glucose	1.425	418.6	26.0	0.001370	0.5735	40.24	40.00
	1.425	415.7	26.0	70	5695	39.97	
	1.425	412.2	25.3	74	5664	39.75	
	1.425	414.0	25.9	71	5676	39.83	
	1.425	418.1	26.1	69	5724	40.17	
	1.425	415.3	26.2	69	5685	39.90	
Alanine	1.4134	419.2	25.3	74	0.5760	40.71	40.42
	1.4134	417.4	25.7	72	5726	40.48	
	1.4134	416.3	25.9	71	5708	40.34	
	1.4134	407.4	21.8	93	5675	40.12	
Cholesterol	0.5848	358.3	25.6	72	0.4916	84.06	83.87
	0.5848	358.9	26.1	69	4913	84.02	
	0.5848	358.3	26.2	69	4905	83.87	
	0.5848	357.9	26.2	69	4900	83.77	
	0.5848	358.6	26.5	67	4902	83.82	

* The factors are those of Table I multiplied by the correction factor 1.003, which is obtained both from the *a* value of 2.006 and from the mean of the glucose analyses. The glucose and alanine samples were aliquots of 1 cc. each of water solutions delivered from a calibrated pipette, from which the deliveries of aqueous solutions were usually exact within 0.002 cc. The cholesterol was dissolved in absolute alcohol. The samples of 1 cc. were measured in a pipette calibrated "to contain." After delivery the pipette was rinsed with alcohol, the rinsings being run into the combustion tube with the sample. The alcohol was removed as described on p. 519. The time of boiling was 1 to 2 minutes.

MACROCOMBUSTION

In this analysis the CO_2 pressure is measured when the mercury level in the chamber is at the 50 cc. mark. Since the volume of solution over the mercury is 4 cc., the pressure is measured with the gas at 46 cc. volume. The analysis is always carried out as in the "Alternative rapid analysis without reabsorption of CO_2" described for the microanalysis on p. 527. The large amounts of

CO_2 can be measured with accuracy without the added step of reabsorption, and the latter is rather undesirable because the amount of heat generated by neutralization of the amount of lactic acid present would raise the temperature of the chamber measurably with each analysis. Because reabsorption is omitted, the analysis is rapid; five can be carried out in the course of an hour.

Apparatus

The apparatus is the same used for the microcombustion, except that the combustion tube is of 25 cc. instead of 15 cc. capacity, and the cup *F* (Fig. 1) is of 5 instead of 2 cc. capacity.

Reagents—

Chromic combustion fluid. This is made the same as for the microcombustion, except that instead of 25 gm. of CrO_3 in 500 cc. of the fluid 30 gm. are used.

Alkaline hydrazine solution for absorbing CO_2. Per 100 cc. of solution to be made, one weighs 6 gm. of hydrazine sulfate into a volumetric flask, and fills the flask to the mark with CO_2-free 3.00 N NaOH of minimal CO_2 content. The details of preparing and handling the solution are the same as for the alkaline hydrazine solution used for the microanalysis.

Approximately 5 N lactic acid. Concentrated lactic acid of specific gravity 1.20 is diluted to 2 volumes with water, and adjusted to 5 N concentration after a preliminary titration of a 50-fold diluted aliquot against 0.1 N NaOH, with phenolphthalein indicator.

Procedure

The preparation of the sample and the combustion tube are the same as described for microcombustions, except for the larger size of the samples, which should contain 8 to 15 mg. of carbon. Of KIO_3 300 instead of 200 mg. are added. In cup *F* (Fig. 1) one places enough combustion fluid to permit delivery of 5 cc. for the combustion.

The *preliminary ejection of air* is carried through *three* successive times, so that nearly all the air in the system is removed.

The *measurement of 2 cc. of alkaline hydrazine solution into the chamber* is carried out as in the microanalyses.

The *combustion, absorption of CO_2 by alkali in the gas chamber,* and *ejection of unabsorbed gases* are likewise carried out as described for microanalyses, except that 5 cc. of combustion fluid are used, and the boiling after the pressure reaches 600 mm. is routinely continued for *3 minutes*, instead of 1.5 minutes. The amount of carbon to oxidize is 5 times as great as in the microcombustion, while the chromic acid is only 3 times as much. The smaller relative excess of reagent seems to necessitate a longer oxidation. More CrO_3 cannot be used in the macroanalysis, as too much O_2 would be evolved for the present apparatus to hold.

For *extraction of CO_2* one measures 2 cc. of 5 N lactic acid into the chamber from an accurate stop-cock pipette, as described for the corresponding addition of 1 cc. of acid in the microanalysis. The extraction of CO_2 is carried out in the same manner, with adjustment of the mercury meniscus in the chamber exactly at the 50 cc. mark, during the last 1.5 minutes of the extraction.

The *p_1 reading* is made with the mercury still at the 50 cc. mark. This completes the analysis.

The p_0 is the p_1 value determined in blank analyses at the same temperature. Correction of effect of temperature change on p_0 is made as described for the "rapid" microanalysis.

For the *blank analysis* only 2 cc. of chromic acid combustion fluid are used; 5 cc. would evolve too much oxygen gas, when none is consumed for combustion.

Calculation for Macrocombustion

The pressure of CO_2 is calculated as $P_{CO_2} = p_1 - p_0$. The mg. of carbon in the sample are calculated by multiplying P_{CO_2} by the proper factor from Table I. The factors of Table I are tested by trial combustions of glucose or other pure substance, as described for the microcombustion, and are corrected if necessary by the b factor found.

Derivation of Formula for Macro Carbon Calculation

The basic formula for the calculation is Equation 1, but it is simplified by the fact that the i factor disappears (there being no opportunity for reabsorption of CO_2 after its extraction), and the value of a is $A - S$. Whence one obtains

$$\text{(6)} \qquad \text{Factor} = \frac{0.0007099\,[A - S(1 - \alpha'_{\text{lactate}})]}{1 + 0.00384t}$$

In the macroanalysis A is 50 cc., and S is 4. In the concentrated acid lactate solution from which the CO_2 is extracted the solubility of CO_2 at 20° is only 56 per cent of the solubility in water. For the range 17–32° the solubility, by the method of Van Slyke (11), has been found to be indicated by the formula

(7) $$\alpha'_{\text{lactate}} = \alpha'_{H_2O}(0.473 + 0.0046t)$$

With the above values substituted for the constants in Equation 6 we obtain

(8) $$\text{Factor} = \frac{0.03266 + (0.00134 + 0.00013t)\alpha'_{H_2O}}{1 + 0.00384t}$$

TABLE V

Macrocombustions

A = 49.95 cc., S = 4.00 cc., a = 45.95 cc., i = 1.000. Duration of boiling, 3 minutes at about 600 mm.

Substance	Sample	P_{CO_2} at 45.95 cc.	Temperature	Factor*	Carbon		
					Found		Theoretical
	mg.	*mm.*	*°C.*		*mg.*	*per cent*	*per cent*
Glucose	37.56	476.7	21.5	0.03149	15.01	39.96	40.00
	39.20	501.3	22.2	139	15.74	40.14	
	35.92	475.4	31.9	016	14.34	39.92	
Alanine	39.73	516.0	23.7	120	16.10	40.52	40.44
	38.75	505.8	24.1	115	15.76	40.66	
Cholesterol	16.098	436.2	25.2	100	13.52	83.99	83.87
	15.020	408.1	25.7	092	12.62	84.01	
Palmitic acid	18.74	450.3	24.5	109	14.00	74.71	74.93
	19.13	462.9	25.2	100	14.35	75.01	

* To correct for the deviation of the A mark on the chamber by 0.05 cc. from the 50 cc. value for which the factors in Table I are calculated, the factors from Table I are multiplied by 45.95/46.00 = 0.999. The results of the analyses calculated with these factors are sufficiently close to theoretical to obviate the necessity of a b correction.

Results with Macrocombustion

Representative analyses are shown in Table V. The macroanalysis gives the same results as the microanalysis (Table III), except that with fatty acids the macroanalysis tends to be a little low, as in the first analysis of palmitic acid in Table V. The smaller excess of CrO_3 in the macroanalysis is the presumable cause.

SUMMARY

A combustion mixture of fuming sulfuric, phosphoric, chromic, and iodic acids is described which gives theoretical yields of CO_2 with types of organic substances that have hitherto been found resistant to wet combustion. The mixture used effects complete oxidation in 1 to 3 minutes.

Procedures are described in which the CO_2 is collected and measured in the Van Slyke-Neill manometric apparatus, the same apparatus serving for submicrocombustions with 0.3 to 0.7 mg. of carbon, microcombustions with 2 to 3.5 mg., and macrocombustions with 8 to 15 mg. The accuracy of the micro- and macroprocedures is equal to that of dry combustions. An analysis can be completed in 20 minutes.

No modification of the method is required for the presence of nitrogen, sulfur, halogen, alkali metals, or, so far as has been ascertained, any other of the substances that interfere with or require modification of the dry combustion.

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MICRODETERMINATION OF SULFATE AND PHOSPHATE BY MANOMETRIC COMBUSTION OF THEIR ORGANIC PRECIPITATES

By CHARLES L. HOAGLAND

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

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The development by Van Slyke and associates (1) of a simple and rapid micromethod for determination of carbon has made possible the measurement of minute amounts of organic precipitates by determination of their carbon content. The first application to mineral analysis was made by Kirk (2), who determined inorganic phosphate by combustion of the strychnine phosphomolybdate precipitate, 0.01 mg. of inorganic phospnorus sufficing for an accurate determination.

In this paper is described a procedure for the microdetermination of sulfate by combustion of the benzidine sulfate precipitate. By performing precipitation, washing, and combustion in a special combustion-centrifuge tube, the procedure is made simple and immune to the losses that endanger transfer operations. It was found that the same tube and technique could be used to simplify Kirk's phosphorus method (2).

The benzidine sulfate precipitate, $C_{12}H_8(NH_2)_2 \cdot H_2SO_4$, contains 12 carbon atoms for 1 of sulfur. Hence when the CO_2 pressure is measured with the gas at 2 cc. volume (submicrocombustion) (3), where the optimal amounts of carbon for analysis are 0.3 to 0.7 mg., the corresponding amounts of sulfur are 0.07 to 0.16 mg. The error of analysis averages 1 part in 200.

However, the amount of sulfur determined may be decreased to as little as 0.02 mg., without greatly increasing the error.

In the other direction, the above upper limits of sample size may be increased 5-fold by using instead of the submicrocombustion, the microcombustion, in which the CO_2 pressure is measured

with the gas at 10 cc. instead of 2 cc. volume. The error of analysis with these larger amounts is less than 1 part in 200.

Apparatus

The apparatus includes that described by Van Slyke and Folch (3) for combustion, and in addition a special centrifuge-combustion tube, which is essentially the standard Van Slyke combustion

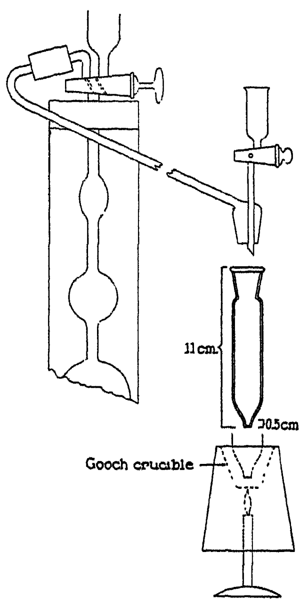

Fig. 1. Diagram illustrating special centrifuge-combustion tube in position ready for attachment to the Van Slyke-Neill manometric apparatus.

tube drawn out conically at the bottom, ending in a shallow capillary. This capillary, 2 mm. in inner diameter and 5 mm. deep, retains suitable precipitates by capillarity, a small column of fluid blocking loss of the precipitate when the supernatant is decanted. The tube is of Pyrex glass. Breaking of the capillary during combustion is prevented by placing over the flame a small Gooch crucible in which the capillary bottom of the combustion tube rests (Fig. 1).

Reagents—

The reagents are those of the Van Slyke-Folch combustion (3), and in addition the following.

Benzidine hydrochloride solution. 4 gm. of benzidine hydrochloride are made up to 250 cc. with 0.2 N HCl.

Acetone, absolute, reagent quality.

90 per cent acetone. 9 volumes of acetone are mixed with 1 volume of water.

Procedure

The conditions for satisfactory precipitation of pure benzidine sulfate in filtrates from biological material have been defined by Fiske (4) and by Stadie and Ross (5). The pH may range from 7 to as acid as 1.6 (5). Phosphate must be absent, or benzidine phosphate is likely to be precipitated with the benzidine sulfate. For each mole of sulfate present 1.4 or more moles of benzidine should be present to make precipitation complete. Chloride should not be present in amounts such that the weight ratio, Cl:S, exceeds 30, or precipitation of benzidine sulfate may not be complete. Acetone added to the aqueous sulfate solution decreases the solubility of the benzidine sulfate so that minute amounts can be precipitated completely.

Preparation of Sulfate Solution—Any method of preparing the sulfate solution which does not interfere with these conditions will presumably be applicable. The procedure of Hoffman and Cardon (6) for removing proteins and phosphate from biological fluids has been found to yield blood filtrates which are satisfactory for analysis by the present method. In Hoffman and Cardon's method (6) phosphate, protein, and fat are removed with ferric chloride and heat. The excess iron is removed with an ammonium hydroxide-ammonium acetate buffer. Centrifugation of the protein-fat coagulum has been found advisable before filtration of the supernatant. The supernatant solution may then be filtered readily through Whatman No. 42 filter paper without appreciable evaporation of acetone from the filtrate before aliquots for precipitation of sulfate are taken. When this technique was employed in the preparation of blood filtrates, it has been possible to recover quantitatively known amounts of inorganic sulfate added to blood before precipitation (Table IV).

For preparation of urine filtrates the method of Fiske (4) for removal of phosphates has been found satisfactory.

Of the prepared solution 2 cc. are to be used for analysis. This volume should therefore contain from 0.02 to 0.16 mg. of sulfate, calculated as S, if the submicrocombustion is to be used, and 0.4 to 0.8 mg. if the microcombustion is to follow.

Precipitation of Benzidine Sulfate—To 2 cc. of protein and phosphate-free filtrate add 1 cc. of acetone, followed by 1 cc. of benzidine hydrochloride reagent added dropwise.

As a check on reagents, control tubes are set up, with water in place of the filtrate. The control serves for a blank analysis, which gives a correction covering all the reagents. The P_{CO_2} value obtained in the blank is the c correction of the analysis.

The combustion tubes, covered to prevent contamination by dust, are set aside in the ice box for complete precipitation. A precipitate begins to form at once and is complete in 1 hour.

Washing—The material is centrifuged 5 minutes at 2000 R.P.M. after which time the precipitate will be packed tightly within the capillary portion of the tube. The supernatant fluid is decanted completely, and the tube is allowed to drain for 10 minutes in a rack on a lint-free towel. 4 cc. of 90 per cent acetone are added to each tube, the inside of the tube, including the ground glass connection area, being washed down completely. The precipitate is not disturbed. Mixing of precipitate with acetone during the washing was found to be entirely unnecessary. Indeed, it contributes to the difficulty of quantitative reclamation of the precipitate by centrifugation. The tube after another 5 minutes of centrifugation and another decantation of supernatant fluid is again allowed to drain as before. This procedure is repeated once more, after which the tubes are placed in a boiling water bath for 30 minutes to drive off all traces of acetone, which if incompletely removed add significantly to carbon combustion values.

Combustion—The material is now ready for combustion. This is carried out as described by Van Slyke and Folch (3). A blank analysis is also carried out in which water replaces the sulfate solution.

Calculation—The pressure, P_{CO_2}, of CO_2 from the burned benzidine is calculated as

$$P_{CO_2} = p_1 - p_2 - c$$

p_1 and p_2 are the manometer readings taken before and after absorption of the CO_2, as described by Van Slyke and Folch (3). c is the value of $p_1 - p_2$ found in the blank analysis.

The sulfate sulfur in the sample is calculated as

$$\text{Mg. S} = P_{CO_2} \times \text{factor}$$

The values of the factor are given in Table I. The factors have been calculated from Table I of Van Slyke and Folch (3) on the assumption that the atomic ratio of carbon to sulfur in the benzidine sulfate precipitate is 12:1, with a corresponding weight ratio of 4.495:1, 12.01 and 32.06 being taken as the atomic weights of C and S. These ratios have been experimentally substantiated by analyses of standard sulfate solutions.

Phosphorus

The procedure is the same as that of Kirk (2), except that the precipitation and washing of the precipitate are carried out in the combustion-centrifuge tube shown in Fig. 1, that combustion is carried out in the same tube, and that the combustion procedure used is the improved one of Van Slyke and Folch (3).

Up to the washing of the precipitate the procedure is the same as Kirk's. The precipitate is now washed with 1 per cent nitric acid by centrifugation with the same technique described for washing the benzidine sulfate precipitate. For the washing two successive portions of 2 cc. each of 1 per cent nitric acid are used.

In the combustion it has been found desirable to alter the oxidation mixture of Van Slyke and Folch (3), since its phosphoric acid content makes prolonged washing of the tubes in flowing water necessary before they can be used again for micro phosphorus determinations. According to a personal suggestion of Van Slyke and Folch, the 100 mg. of KIO_3 are added, as in the usual combustion, but instead of the usual 2 cc. of chromic-sulfuric-phosphorus acid mixture, 2 cc. of reagent grade of concentrated sulfuric acid (sp. gr. 1.34) are added. Strychnine burns rather easily, and the iodic acid alone suffices. Chromic acid in pure sulfuric acid has the added disadvantage of depositing insoluble precipitates in the capillary of the combustion tube. The boiling is continued gently for $3\frac{1}{2}$ minutes. Except for these two changes the combustion is carried out as described by Van Slyke and Folch (3).

TABLE I

For Calculation of Phosphorus from P_{CO_2} Obtained by Combustion of Strychnine Phosphomolybdate Precipitate and Sulfur from P_{CO_2} Obtained by Combustion of Benzidine Sulfate Precipitate

Temperature	Factors by which P_{CO_2} is multiplied to give		
	Mg. phosphorus	Mg. sulfur	
	Submicroanalysis $a = 2.00$ $S = 3.00$	Submicroanalysis $a = 2.00$ $S = 3.00$	Microanalysis $a = 10.00$ $S = 3.00$
°C.			
10	0.00005190	0.0003279	0.001624
11	63	61	16
12	35	44	08
13	10	28	00
14	5085	12	1592
15	60	3197	85
16	36	82	77
17	12	67	69
18	4989	52	62
19	66	37	54
20	43	21	47
21	20	07	40
22	4897	3093	33
23	75	79	26
24	53	65	19
25	32	51	12
26	11	38	05
27	4790	25	1499
28	70	12	93
29	50	00	86
30	30	2988	80
31	20	76	74
32	4691	64	68
33	72	52	62
34	53	40	57
35	34	28	51

TABLE II

Recovery of Carbon by Wet Combustion of Benzidine Precipitated by Known Quantities of Sulfate

Source of sulfate	Quantity of S	C recovered in benzidine sulfate ppt.	Recovery ratio $\frac{\text{mg. atoms C}}{\text{mg. atoms S}}$
	mg.	*mg.*	
$(NH_4)_2SO_4$	0.02	0.0901	12.1
	0.02	0.0887	11.8
K_2SO_4	0.02	0.0899	12.0
	0.02	0.0888	12.0
$(NH_4)_2SO_4$	0.04	0.1785	11.9
	0.04	0.1788	11.9
K_2SO_4	0.04	0.1812	12.1
	0.04	0.1798	12.0

TABLE III

Recovery of Known Quantity of Sulfate by Wet Combustion of Benzidine Sulfate Precipitate (Submicrocombustions)

S in sample	P_{CO_2}	$P_{CO_2} \times f_S$	Deviation from theoretical
mg.			*per cent*
0.10	320.3	0.0992	−0.8
0.10	329.9	0.1000	0.0
0.08	265.8	0.0808	+1.0
0.08	263.5	0.0802	+0.2
0.05	164.0	0.0497	−0.6
0.05	165.6	0.0499	+0.4
0.04	131.0	0.0397	−0.7
0.04	129.0	0.0398	−0.5
0.03	100.0	0.0302	+2.0
0.03	97.3	0.0295	−1.6
0.02	65.3	0.0198	−1.0
0.02	67.3	0.0204	+2.0

TABLE IV

Recovery by Gasometric Method of Sulfate Added to Human Plasma

Plasma sample No.	Sulfate S found	Sulfate S added	Sulfate S recovered	Per cent recovery
	mg. per cent	*mg. per cent*	*mg. per cent*	
1	1.4	1.0	2.35	98.0
2	1.8	2.0	3.90	97.3
3	0.9	2.5	3.45	101.5
4	2.1	3.0	5.00	98.1

Calculation—As in the sulfur analysis, the pressure, P_{CO_2}, of CO_2 from the carbon in the precipitate is calculated as

$$P_{CO_2} = p_1 - p_2 - c$$

where c is the value of $p_1 - p_2$ obtained in a blank analysis. The value of P_{CO_2} is multiplied by the proper factor from Table I to calculate mg. of phosphorus. The phosphorus factors of Table I are obtained by dividing the carbon factors of Van Slyke and Folch (3) by 28.4, which was found by Kirk (2) to be the weight ratio, C:P, in the strychnine molybdate precipitate. The factors of Table I are slightly different from those of Kirk, for the reason that the S and α' values in the Van Slyke-Folch combustion, and consequently the carbon factors, are slightly different from those of the earlier combustion (1).

Results

In order to test the stoichiometric relationship between the benzidine carbon and known concentrations of inorganic sulfate, solutions of sulfate containing sulfur in amounts ranging from 0.1 to 0.02 mg. were precipitated with the benzidine chloride reagent; the carbon in the precipitate was determined by the method outlined above, and calculated as sulfur from the factors given in Table I. This served at the same time as a check on the accuracy of the factors.

For quantities of sulfur ranging from 0.04 mg. upwards the average deviation from theoretical results was about 1 part in 200 (Tables II and III). Even with the smaller samples ranging from 0.04 to 0.02 mg. the error is rarely more than 2 per cent. These recoveries are considered adequate for the extremely small amounts of sulfate taken for analysis.

Additional results with biological material will be reported later in publications of experimental work in which the method is being used.

SUMMARY

A method for the microdetermination of inorganic sulfate has been outlined, based on the combustion of the carbon in the

benzidine sulfate precipitate, and measurement of the carbon dioxide in the Van Slyke-Neill manometric apparatus.

A special centrifuge-combustion tube in which all procedures from precipitation to combustion are carried out has been designed to obviate losses that might occur through transfer of the material from tube to tube.

With known inorganic sulfate concentrations the method yields theoretical results. It is applicable to blood and urine filtrates following removal of phosphate.

The centrifuge-combustion tube can also be used to avoid transfers and simplify technique in the manometric micro phosphorus method of Kirk (2).

The author is greatly indebted to Dr. D. D. Van Slyke for his valuable suggestions and helpful criticism.

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MICROMANOMETRIC DETERMINATION OF MAGNESIUM

By CHARLES L. HOAGLAND

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

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The quantitative reaction which occurs in alkaline solution between the magnesium ion and 8-hydroxyquinoline is well known (1, 2).

$$Mg^{++} + 2C_9H_6NOH = Mg(C_9H_6ON)_2 + 2H^+$$

Several attempts to utilize this reaction for the quantitative estimation of magnesium are recorded. The phenolic properties of the 8-hydroxyquinoline complex have been used to develop with Folin's reagent (3) a colorimetric method for the estimation of magnesium (1). A method based on the development of a green color by iron with the magnesium-hydroxyquinoline complex has been used by Lavollay (4). Others have resorted to diazotization and conversion of the magnesium-hydroxyquinoline complex to a dye by coupling with diazobenzenesulfonic acid (5). Methods by which the magnesium is precipitated with 8-hydroxyquinoline and determined gravimetrically (6) have been in use industrially for some time, particularly in water analysis. Before the determination is carried out, precautions must be taken to remove aluminum, manganese, copper, and zinc if these are present in sufficient amounts to cause precipitation of the reagent. These metals rarely exist in biological materials in sufficient concentration to necessitate removal before precipitation of magnesium. Iron in small amounts does not precipitate at the alkaline pH which is optimum for magnesium (4).

Because of the simplicity of the technique involving direct precipitation of magnesium-hydroxyquinoline, an attempt has been made to improve its sensitivity and obviate colorimetry and standards by substituting direct combustion of the hydroxyquino-

line complex by the method of Van Slyke and Folch (7). Because the precipitate contains 18 carbon atoms to 1 magnesium, accurate determination requires only 0.03 mg. of Mg.

In the determination of magnesium use is made of a special combustion tube described elsewhere in this *Journal* in connection with the gasometric determination of sulfate (8).

Reagents—

8-Hydroxyquinoline, C.P., 2 per cent solution in 95 per cent alcohol.

Saturated aqueous ammonium chloride solution.

Ammonia water, concentrated.

The 8-hydroxyquinoline solution on standing loses its property of combining with magnesium. This is associated with a loss of its phenolic properties, as indicated by a failure to give a color with Folin's phenol reagent (9, 10). For this reason the reagent must be prepared fresh daily and discarded should it be found to give a starch-iodide reaction.

Procedure

The sample should contain 0.03 to 0.07 mg. of magnesium, dissolved in 2 to 10 cc. of protein- and calcium-free solution. Place the solution of the sample in the special centrifuge-combustion tube (8); add 0.2 cc. of 8-hydroxyquinoline reagent. Follow with 0.2 cc. of saturated ammonium chloride solution. Rotate vigorously and set aside for 5 minutes. Add 1 to 2 drops of ammonia water. A fine greenish yellow precipitate of the magnesium complex begins to form almost immediately.

Tubes containing 2.0 cc. of water with an identical quantity of reagent are set up at the same time. The value obtained from combustion of this "blank" is subtracted from the value obtained from the test substance.

The tubes are allowed to stand at room temperature for 30 minutes. A portion of the light magnesium precipitate has a tendency to climb up the side of the tube as evaporation of ammonia proceeds. This precipitate is washed down with 0.5 cc. of saturated ammonium chloride solution, after which the precipitate is collected in the capillary of the combustion tube by centrifugation at 2000 R.P.M. for 5 minutes. The supernatant fluid is removed by a fine capillary attached to a suction flask.

If suction is begun only after the capillary is placed under the surface of the supernatant fluid, the trace of magnesium precipitate floating on the surface adheres to the wall of the combustion tube and is not lost. The inside walls of the tube and the precipitate are then washed with 5 cc. of the saturated ammonium chloride solution, care being taken to wash down the entire inside of the combustion tube, including the ground glass connection area. It is not necessary to disturb the precipitate which is packed thinly at the bottom of the small capillary at the tip of the combustion tube. It is, however, essential that all hydroxyquinoline reagent adhering to the inside of the combustion tube be washed away. Following the second washing and removal of supernatant fluid by capillary suction, the tubes are placed in a boiling water bath for 30 minutes to remove traces of moisture from the precipitate. The precipitate is then oxidized with chromic acid reagent, and the carbon dioxide measured in the Van Slyke-Neill manometric apparatus according to the present technique of Van Slyke and Folch for the determination of carbon in organic compounds (7).

Calculation—The pressure, P_{CO_2}, of the CO_2 from the burned precipitate is calculated as

$$P_{CO_2} = p_1 - p_2 - c$$

where p_1 and p_2 are the manometer readings before and after absorption of the CO_2 as described by Van Slyke and Folch (7) and c is the value of $p_1 - p_2$ found in blank analyses.

The amount of magnesium in the sample, in mg., is calculated by multiplying P_{CO_2} by the proper factor in Table I. The factors in Table I are obtained by multiplying the carbon factors of Van Slyke and Folch (7) by the weight ratio, Mg : 18C = 24.32 : 216.18 = 0.1125.

Recovery of Theoretical Carbon from Magnesium-8-Hydroxyquinoline Complex—From the formula, $Mg(C_9H_6ON)_2$ (6), for each magnesium atom 18 carbon atoms should be available for combustion to carbon dioxide. In order to test the completeness of precipitation and combustion known quantities of Mg^{++} in the form of $MgSO_4 \cdot 7H_2O$ and $MgCl_2 \cdot 6H_2O$ were precipitated by the technique outlined above and the precipitates were subjected to combustion. Table II indicates that with amounts of Mg^{++}

varying from 0.010 to 0.040 mg. a stoichiometric relationship of 1 atom of Mg^{++} to 18 carbon atoms accurate within the range of experimental error may be expected. In amounts less than 0.020,

TABLE I

For Calculation of Magnesium from P_{CO_2} Obtained by Wet Combustion of Magnesium-Hydroxyquinoline Precipitate

Temperature	Factors by which P_{CO_2} is multiplied to give mg. magnesium $a = 2$ $S = 3$
°C.	
10	0.0001655
11	49
12	40
13	32
14	25
15	17
16	09
17	02
18	1594
19	86
20	79
21	72
22	64
23	57
24	50
25	43
26	37
27	30
28	23
29	17
30	11
31	05
32	1499
33	93
34	86
35	80

a very slight solubility of the hydroxyquinoline complex may be expected to yield upon combustion slightly less than the theoretical carbon.

In Table III are given values obtained from the precipitation of known quantities of magnesium.

TABLE II

Recovery of Carbon by Wet Combustion from Precipitate of Magnesium with 8-Hydroxyquinoline

Source of Mg^{++}	Mg^{++} taken	Carbon found	Recovery ratio Mg:C
	mM	*mM*	
$MgCl_2 \cdot 6H_2O$	0.000412	0.00725	1:17.6
$MgSO_4 \cdot 7H_2O$	0.000412	0.00725	1:17.6
$MgCl_2 \cdot 6H_2O$	0.000823	0.01481	1:18.0
$MgSO_4 \cdot 7H_2O$	0.000823	0.01486	1:18.05
$MgCl_2 \cdot 6H_2O$	0.001650	0.02937	1:17.8
$MgSO_4 \cdot 7H_2O$	0.001650	0.02993	1:18.2

TABLE III

Recovery of Known Concentrations of Magnesium Calculated from Carbon Dioxide Obtained from Combustion of Magnesium-Hydroxyquinoline Complex

Mg^{++} taken	Mg^{++} found	Deviation from theoretical
mg.	*mg.*	*per cent*
0.01	0.0098	−2.0
0.02	0.0199	−0.5
0.03	0.0300	0.0
0.04	0.0400	0.0
0.05	0.0502	+0.4

TABLE IV

Recovery of Magnesium Added to Serum

Sample No.	Serum magnesium	Magnesium added	Total magnesium expected	Total magnesium recovered	Per cent recovery
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
1	2.8	2.0	4.8	4.56	96.7
2	4.1	3.0	7.1	6.96	97.2
3	3.1	4.0	7.1	7.00	98.6
4	2.5	5.0	7.5	7.20	96.0

Determination of Magnesium in Biological Materials—A variety of satisfactory techniques exists for the preparation of blood and serum filtrates suitable for magnesium determination and will not be repeated in detail here (10–12). On a calcium-free filtrate

the above procedure is carried out and magnesium expressed in mg. computed from the P_{CO_2} factors given in Table I. In this laboratory the method of Berg, Walker, and Skopp (13), cited by Snell and Snell (12), has proved particularly satisfactory.

Table IV summarizes the results obtained in an attempt to recover known concentrations of Mg^{++} added to sera before removal of proteins and calcium. From 96.0 to 98.6 per cent recovery was obtained.

For urine, calcium is satisfactorily removed as the oxalate by the method of McCrudden (14), after which the magnesium is precipitated from an aliquot of the filtrate by the technique described above.

SUMMARY

A micromanometric technique for the determination of magnesium is outlined whereby the carbon in the magnesium-hydroxyquinoline complex is oxidized to carbon dioxide and measured in the Van Slyke-Neill manometric apparatus. The method yields theoretical results with known magnesium concentrations which compare well with theoretical values, and is applicable to the determination of magnesium in blood and urine filtrates.

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THE SPECIFICITY OF SALMON PEPSIN

Sirs:

Norris and Elam[1] have described the isolation, in crystalline form, of a protein-digesting enzyme from the stomachs of Pacific Coast king salmon. It seemed desirable to determine whether this salmon pepsin was similar in specificity to swine pepsin. Previous work[2] had shown that crystalline swine pepsin hydrolyzed simple compounds containing glutamic acid and tyrosine, such as carbobenzoxy-*l*-glutamyl-*l*-tyrosine, glycyl-*l*-glutamyl-*l*-tyrosine, etc. It was further found that the above substrates for swine pepsin were also hydrolyzed by concentrated pepsin

Substrate	pH of test solution	Increase in amino N per cc. test solution	Hydrolysis*
		mg.	*per cent*
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine	3.2	0	0
	4.2	0	0
Glycyl-*l*-glutamyl-*l*-tyrosine	3.2	−0.01	0
Carbobenzoxyglycyl-*l*-glutamyl-*l*-tyrosine	3.2	−0.01	0
	4.6	0	0
Benzoyl-*l*-arginineamide	3.2	0†	0
Benzoyl-*l*-tyrosylglycineamide	3.2	0.04	6
	5.6	0.027	4
Edestin	3.7	0.66	

Enzyme concentration, 1.45 mg. of protein N per cc. of test solution; concentration of synthetic substrates, 0.05 mM per cc. of test solution; edestin concentration, 18 mg. per cc. of test solution; time of incubation, 48 hours; temperature, 40°.

* The 100 per cent hydrolysis of one peptide linkage results in an increase of 0.70 mg. of amino nitrogen per cc. of test solution.

† This value was obtained by microtitration of carboxyl groups.

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preparations from beef, sheep, and chicken.[3] Through the kindness of Dr. Norris and Dr. Elam, who provided us with a sample of crystalline salmon pepsin, it was possible to subject a number of the substrates to the action of this enzyme. It will be noted (*cf.* the table) that the compounds carbobenzoxy-*l*-glutamyl-*l*-tyrosine, glycyl-*l*-glutamyl-*l*-tyrosine, and carbobenzoxyglycyl-*l*-glutamyl-*l*-tyrosine were not attacked by the enzyme preparation. However, under similar conditions, edestin was extensively hydrolyzed. It would appear, therefore, that salmon pepsin differs markedly in specificity from the pepsins of swine, beef, sheep, and chicken.

The following compounds were also subjected to the action of salmon pepsin: benzoyl-*l*-arginineamide (substrate for crystalline beef trypsin) and benzoyl-*l*-tyrosylglycineamide (substrate for crystalline beef chymotrypsin). The first of these was completely resistant to the enzyme, while the latter showed a very slight hydrolysis.

Laboratories of The Rockefeller Institute for Medical Research
New York

JOSEPH S. FRUTON
MAX BERGMANN

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[3] Bergmann, M., and Fruton, J. S., unpublished work.

THE BIOLOGICAL CONVERSION OF ORNITHINE INTO PROLINE AND GLUTAMIC ACID

Sirs:

Deutero ornithine, when given to normal adult mice in addition to the ordinary stock diet, is rapidly and extensively converted into deutero arginine, and the latter is introduced into the animals' proteins.[1] Proline and glutamic acid have now been isolated from the same hydrolysate of mouse proteins as had yielded deutero arginine. Proline was precipitated as the rhodanilate[2] and purified by conversion into the hydantoin.[3] This contained 0.31 ± 0.03 atom per cent deuterium, indicating that ornithine was partly converted into proline, and the latter introduced into the protein linkage.

The glutamic acid isolated contained 0.07 ± 0.01 atom per cent deuterium. The compound was degraded with chloramine-T; the resulting barium succinate[4] contained 0.16 ± 0.01 atom per cent deuterium. As no deuterium was lost during degradation, none could have been present in the α position of glutamic acid. The values do not indicate how much ornithine was converted into the two amino acids, as we do not yet know what fraction of the deuterium was lost during the conversions or the extent to which the new amino acids were mixed with their non-isotopic analogues. The dietary casein and the animals' proteins contain a very large amount of (non-isotopic) glutamic acid. The newly formed isotopic amino acid must have mixed with the glutamic acid originating from these sources, whereby the isotope would have been considerably diluted. Even if a large part of the ornithine had been converted into glutamic acid, the isotope concentration of the latter could not have been high.

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Krebs[5] has recently put forward evidence on the basis of work with *d*-amino oxidase that proline, ornithine, and glutamic acid are metabolically interlinked.

The present experiment, carried out with normal animals on their normal diet, shows that the conversion of ornithine into arginine, proline, and glutamic acid and the introduction of these compounds into the proteins are continuous processes.

Department of Biochemistry
College of Physicians and Surgeons
Columbia University
New York

Marjorie Roloff
S. Ratner
Rudolf Schoenheimer

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INHIBITION OF FLAVOPROTEIN OXIDATIVE CATALYSIS BY SUBSTITUTED PHENOLS

Sirs:

Certain phenols having nitro and halogen groups as substituents exhibit two types of effect on cell respiration. The first consists in a stimulation of oxygen consumption, produced at relatively low substituted phenol concentrations. The second effect, produced at relatively higher substituted phenol concentrations, consists in an inhibition of a fraction of the respiration; this is accompanied, in fertilized *Arbacia* eggs, by a reversible inhibition of cell division[1] and, in various tumor tissues, by a specific inhibition of the Pasteur effect.[2] This communication reports the results of preliminary efforts to identify the respiratory process or processes inhibited by the substituted phenols.

In numerous experiments performed from 1935 to date, it was found that 4,6-dinitro-*o*-cresol, in concentrations up to 1000 times the physiologically active concentrations, had no effect on a number of metal-containing oxidative systems.[1] The substituted phenols were also virtually without effect on a number of dehydrogenase systems. Meanwhile, evidence had accumulated from experiments on living cells to indicate that the substituted phenols might, in appropriate concentrations, inhibit some intermediate carrier process, possibly a flavoprotein catalysis. Independent chemical evidence for this suggestion came from the fact that riboflavin had been found[3] to have a high solubility in, and affinity for, phenol and certain phenol derivatives.

In the present investigation a number of physiologically active substituted phenols, including 4,6-dinitro-*o*-cresol, 2,4-dinitro-*o*-cyclohexylphenol, and 2,4,5-trichlorophenol, have been found

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to inhibit two typical flavoprotein catalysts, *d*-amino acid oxidase[4] and heart muscle flavoprotein;[5] however, these agents did not inhibit milk xanthine oxidase, an enzyme for which there is some, as yet equivocal, evidence[6] for participation of a flavin group. A physiologically inactive substituted phenol, *o*-nitrophenol, was without effect (up to 10^{-2} M) on any of these enzyme systems. In representative experiments with *d*-amino acid oxidase, oxygen consumption measurements were made by the direct Warburg method in air at 30°. The effects of variation in pH, flavin-dinucleotide concentration, protein concentration, substrate concentration, and substituted phenol concentration were studied. The results of a typical experiment with varying substrate con-

Concentration of *dl*-alanine, moles per liter	O_2 consumed in 1 hr., c.mm.		Per cent inhibition
	In control	In 10^{-3} M 2,4,5-trichlorophenol	
0.1	232	113	51
0.05	185	73	60
0.025	141	44	69
0.0125	101	20	80
0.0063	72	14	81
0.0032	43	7	84

centration are shown in the table. The final concentration of flavin-dinucleotide[4] was 0.025 mg. per ml., that of the protein[7] 0.15 mg. per ml. The total volume in each flask was 5.5 ml.; the pH was 8.3.

Lilly Research Laboratories
Indianapolis

M. E. Krahl
A. K. Keltch
G. H. A. Clowes

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TRANSAMINATION WITH PURIFIED ENZYME PREPARATIONS (TRANSAMINASE)

BY PHILIP P. COHEN*

(*From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*)

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Braunstein and Kritzmann (1) have reported that with pigeon breast muscle any α-amino acid, with the possible exception of glycine, is active in transamination with either α-ketoglutaric or oxaloacetic acid. On the other hand, the author (2) found that transamination in pigeon breast muscle is limited to the following reactions.

$$(1)\quad l(+)\text{-Glutamic acid} + \text{oxaloacetic acid} \underset{b}{\overset{a}{\rightleftharpoons}} \alpha\text{-ketoglutaric acid} + l(-)\text{-aspartic acid}$$

$$(2)\quad l(+)\text{-Glutamic acid} + \text{pyruvic acid} \underset{b}{\overset{a}{\rightleftharpoons}} \alpha\text{-ketoglutaric acid} + l(+)\text{-alanine}$$

Attempts to find evidence for a third reaction,

$$(3)\quad l(-)\text{-Aspartic acid} + \text{pyruvic acid} \underset{b}{\overset{a}{\rightleftharpoons}} \text{oxaloacetic acid} + l(+)\text{-alanine}$$

were not successful.

Braunstein and Kritzmann (1) further observed that transamination took place between glutamic acid and various α-keto acids in the presence of a purified enzyme preparation from pigeon breast muscle. Kritzmann (3) reported in some detail on the preparation and properties of these enzymes from pigeon breast and pig heart muscle and stated (4) that two distinct enzyme systems were involved, one concerned with the reversible trans-

* National Research Council Fellow in Medical Sciences.

amination of glutamic and the other of aspartic acid. On the basis of this, Braunstein (5) suggested the names of *glutamic aminopherase* for the former, *aspartic aminopherase* for the latter. Since the original term *Umaminierung* used by these workers (6) has been accepted with the English (and French) equivalent of *transamination* (5, 7, 8), it is suggested here that the enzyme (or possibly enzymes) catalyzing the transfer of amino nitrogen be termed *transaminase.* The latter term is more euphonious and does not suffer from a mixed etymology. Further it will be shown in this and the next paper that it has not been possible to demonstrate the existence of two separate enzyme systems, one concerned with glutamic and the other with aspartic acid, but rather that the most active transaminase preparations catalyze a reaction in which *both* glutamic and aspartic acids are substrates.

In a recent review of transamination (9) Braunstein reported considerable differences in the percentage transamination of certain amino acids with pigeon breast muscle on the one hand and a purified enzyme preparation on the other. It therefore seemed desirable to study the activity of transaminase with different α-amino acids and α-keto acids. This paper deals with such experiments, in addition to a description of a new analytical method for studying transamination with purified enzyme preparations.

Methods of Studying Transamination with Transaminase

The use of purified enzyme preparations simplifies the study of transamination considerably as compared with sliced or minced tissue. Since the enzyme preparations are free of oxidation systems, it is not necessary to use either anaerobic conditions or inhibitors. Further, the comparative freedom from extraneous material and side reactions in the case of the enzyme preparations makes not only for greater specificity and accuracy of the analytical procedures, but also permits the use of further simple analytical methods not applicable to minced and sliced tissue.

Satisfactory micromethods are now available for glutamic acid (10), α-ketoglutaric acid (11), and oxaloacetic acid (12, 13). No satisfactory specific micromethods have as yet been devised for alanine or aspartic acid. However, a satisfactory method for

measuring the formation and disappearance of aspartic acid will be described in the next section.

Procedures and Methods

Preparation of Transaminase—Fresh pigeon breast and pig heart muscle were used as a source of transaminase. The method of preparation from both sources is essentially the same and followed that of Kritzmann (3). Pig hearts were obtained from the slaughter-house immediately after death and kept on ice while in transit. If not used immediately, the heart muscle can be stored in a solid, frozen state for several weeks and still yield active transaminase preparations. The ventricular tissue was dissected free of fat, coarsely hashed in a meat grinder, and twice washed by suspending in 3 volumes of distilled water. After the second washing the tissue was sucked as dry as possible through muslin on a suction filter and again put through the meat grinder, this time arranged so as to yield a fine mince. A weighed amount of the finely minced tissue was ground up with sand in a large mortar and extracted with 5 volumes of 1 per cent potassium bicarbonate solution, added in small portions during the grinding. The suspension was allowed to stand for 30 minutes with intervals of grinding, and then centrifuged. The supernatant was filtered through a double layer of fine muslin and the pulp returned to the mortar to be reextracted twice with 2 volumes of potassium bicarbonate solution in the above manner. The combined extracts were then incubated at 37° for 1 hour, cooled, and brought to pH 4.2 by the slow addition of 10 per cent acetic acid and 1 M acetic acid-acetate buffer, pH 4.2. The 10 per cent acetic acid solution was added in an amount slightly greater than that required to neutralize the potassium bicarbonate used. The acetate buffer was added in an amount equivalent to one-fifteenth of the volume of the extract. The turbid solution was allowed to stand in the refrigerator overnight. A bulky grayish precipitate is observed on the following morning with a clear, dark brown supernatant. The supernatant was carefully decanted and the remaining suspension centrifuged in the cold room. The centrifugate was repeatedly suspended and centrifuged in about 10 volumes of cold distilled water. (This procedure was repeated six to seven times, each centrifugation

lasting for 45 to 60 minutes before the supernatant was free of turbidity.) The moist precipitate was then preserved in the cold with the addition of several drops of octyl alcohol. By this treatment the moist precipitate remains active for 10 to 14 days. 500 gm. of fresh pig heart muscle yield approximately 50 gm. of moist precipitate which has a dry weight of about 5 gm.

Before the phosphate extract of the stored moist precipitate was prepared, the latter was resuspended in cold distilled water and centrifuged at high speed in the cold. The freshly centrifuged moist precipitate was suspended in 3 to 4 parts of M/15 phosphate buffer, pH 7.7, and kept at 55° for 20 minutes. The suspension was then cooled and centrifuged in the cold for 45 minutes. The supernatant, which was used as the enzyme solution, was usually free of tubidity and has a slight yellowish tinge. The solution now has a pH of 7.5. The protein content of these preparations varied from 1 to 4 mg. per ml., as measured by nitrogen determinations. The enzyme solutions rapidly lose their activity on standing. Attempts to prepare active dry preparations have not been successful to date.

The enzyme preparations showed no oxygen uptake in the presence of methylene blue and the following, *l*(+)-alanine, *l*(+)-glutamic, *l*(−)-aspartic, α-ketoglutaric, oxaloacetic, pyruvic, and succinic acids.

Preparation of Boiled Muscle Extracts—Fresh pig heart and pigeon breast muscle were used. 40 gm. of fresh tissue were finely minced and suspended in 50 ml. of M/15 phosphate buffer, pH 7.7. The suspension was placed in a boiling water bath for 10 minutes and then filtered. The filtrate was brought to a quick boil, cooled, and filtered again. This filtrate was used as boiled muscle extract.

Preparation of Substrates. Oxaloacetic Acid—Oxaloacetic acid was prepared by the hydrolysis of oxaloacetic ester according to Simon (14). The ester was synthesized according to Wohl and Österlin (15). The hydrolysis of oxaloacetic ester with concentrated HCl according to Simon gives low yields of oxaloacetic acid owing to the incompleteness of the hydrolysis. It was found possible to increase the yield considerably by extracting the filtrate from the first hydrolysis mixture with ether, removing the ether, and again adding concentrated HCl to the unhydrolyzed oxalo-

acetic ester. The oxaloacetic acid was recrystallized from benzene-acetone; m.p. 150°, uncorrected. The free acid was found to be stable for many months if stored in a dark bottle in the refrigerator.

α-Ketoglutaric Acid—α-Ketoglutaric acid was prepared by the hydrolysis of oxalosuccinic ester according to Neuberg and Ringer (16). Oxalosuccinic ester was synthesized according to Wisclicenus and Waldmuller (17). The α-ketoglutaric acid prepared in the above manner contained about 5 per cent free oxalic acid. This was removed by adding $CaCl_2$ solution to a warm aqueous solution of the acid, filtering off the precipitate, and extracting the acidified aqueous filtrate with ether. The α-ketoglutaric acid purified in this manner had a melting point of 111–113°, uncorrected.

Amino Acids—Most of the amino acids employed were commercial products. The author is indebted to Dr. Joseph S. Fruton for a sample of *d*(−)-glutamic acid, to Professor Vincent du Vigneaud for a sample of *d*(−)-alanine, and to Dr. Fritz A. Lipmann for a sample of phosphoserine. The samples of *l*(−)-cysteic acid and metal-free *l*(−)-cysteine were obtained from Dr. Abraham White, to whom the author is duly grateful.

Pyruvic Acid—Pyruvic acid was freshly distilled and made up as a 1 M aqueous solution. When stored in a brown bottle in the refrigerator, such a solution remains stable and non-toxic for months. The aqueous solution was neutralized with 1 M sodium bicarbonate solution before being used as substrate.

Experimental Procedure

All substrates were neutralized to the proper pH just before use. The α-keto acids were usually made up as 0.2 M and the amino acids as 0.06 M solutions. (*dl*-Amino acids were made up in twice the concentration used for the *d* or *l* forms.)

The enzyme solutions (from pig heart muscle unless indicated otherwise) and substrates were incubated in stoppered Erlenmeyer flasks. The latter were fixed to a shaking rack which was attached to a constant temperature bath at 38°. Since added substrates were not oxidized in the presence of the enzyme and air, it was not necessary to employ anaerobic conditions. For the determination of aspartic, glutamic, and α-ketoglutaric acids, the reac-

tion was stopped by the addition of 1 ml. of 10 per cent H_2SO_4, followed by 0.5 ml. of 10 per cent sodium tungstate solution. Suitable aliquots of the protein-free filtrates were employed for these analyses, as described later. For the determination of oxaloacetic acid, the reaction was stopped by the addition of 0.5 ml. of 50 per cent citric acid.

Analytical Procedures

Determination of Aspartic Acid Formation and Disappearance—Dakin (18) first observed that in the presence of an excess of chloramine-T aspartic acid yielded 2 moles of CO_2, while most of the other amino acids tested yielded but 1. The application of this reaction to the manometric determination of amino nitrogen was carried out by Dr. H. A. Krebs (personal communication[1]) who concluded that the yields of CO_2 from the different amino acids were not sufficiently constant to be generally used for amino nitrogen determination in place of a reagent like ninhydrin (19). However, while yields of CO_2 vary from amino acid to amino acid, any given amino acid yields a constant amount of CO_2 under defined experimental conditions. Since of the amino acids concerned in transamination aspartic acid gives 2 moles of CO_2 while alanine and glutamic acid give but 1 when treated with chloramine-T, it is apparent from a glance at Reactions 1 and 3 that changes in CO_2 production can be used as a measure of transamination. In view of the fact that a well washed enzyme preparation is employed which is free of non-protein nitrogen, the dèproteinized experimental solutions contain only a mixture of the reactants and thus can be analyzed without difficulty.

In practice the following procedure is adopted. The experiments are so arranged that the control flasks contain the amino acid to be studied plus the enzyme solution. The experimental flasks contain in addition the α-keto acid. After a suitable incubation period the solution is deproteinized by the addition of 1 ml.

[1] The following is a summary of Dr. Krebs' findings: Glutamic acid, alanine, phenylalanine, leucine, proline, N-methylleucine yield 101 to 104 per cent CO_2 (pH 4.7, 40°); leucine, valine, isoleucine yield 105 to 115 per cent; aspartic acid and glycine form rapidly 1 mole of CO_2 and, more slowly, a 2nd mole. Ammonium salts interfere by forming N_2 with chloramine-T.

of 10 per cent H_2SO_4 plus 0.5 ml. of 10 per cent sodium tungstate solution, and diluted to a volume of 10 to 14 ml. before filtering. An aliquot of the protein-free filtrate is then transferred to a graduated tube and the volume noted. (It is desirable to use relatively large aliquots of the order of 5 ml. in order to minimize errors in volume readings.) The tubes are placed in a boiling water bath for 30 to 45 minutes, or until the solutions are concentrated to approximately one-half the original volume. This serves to decompose the oxaloacetic acid which in high concentrations yields CO_2. The tubes are then cooled and the original volume restored with distilled water. (Where small amounts of amino acid are present, the concentrated solution is used.) An aliquot of 1 ml. is then taken for the manometric determination of CO_2.

For the manometric determination of CO_2, conical Warburg vessels with side arms having a capacity of at least 1 ml. are employed. 1 ml. of the solution to be analyzed is pipetted into the side arm. The main compartment contains 1 ml. of citrate buffer solution, pH 4.7 (see (10)), plus 2 ml. of freshly prepared 10 per cent chloramine-T solution. A control vessel contains the same solutions as the experimental vessels, except that 1 ml. of acidified and CO_2-free water is placed in the side arm. The flasks are then attached to the manometers and shaken in a constant temperature bath at 38°. The reaction is usually complete in 20 to 30 minutes. Typical results are shown in Table I.

Determination of Glutamic Acid—Glutamic acid was determined on aliquots of deproteinized solutions according to the author's method (10). However, as pointed out previously, storage of the succinoxidase preparation at temperatures even as low as 4° results in an increasing "blank" oxygen uptake with a decrease in activity. As a result fresh enzyme preparations need to be made frequently. It has been found possible to overcome this difficulty to some extent. In the first place it has been found that freshly dissected pigeon breast muscle will yield active succinoxidase preparations even after 2 months storage, provided that the muscle is kept in a solid frozen state. This is best achieved in the freezing chamber of an electric refrigerator. As a result of this, the need for maintaining live pigeons as a source of succinoxidase is eliminated. Further, it has been found that washed preparations of the pigeon

breast muscle (see (10)), whether prepared from fresh or frozen tissue, will retain their activity with a low blank for as long as 2 weeks, if also kept in the solid frozen state.

Determination of α-Ketoglutaric Acid—α-Ketoglutaric acid was determined according to the method of Krebs (11). An aliquot of the protein-free filtrate was transferred to a Kutscher-Steudel extractor and treated with 1 ml. of 50 per cent H_2SO_4 plus 2 ml.

TABLE I

CO_2 Production from α-Amino and α-Keto Acids with Chloramine-T

Each vessel contains 0.5 ml. of 0.01 M amino or keto acid plus 1 ml. of citrate buffer, pH 4.7, plus 2 ml. of chloramine-T; temperature 38°. (Theoretical CO_2 yield calculated on the basis that 0.5 ml. of 0.01 M amino acid yields 112 microliters of CO_2.)

Amino acid	CO_2 found	Theoretical
	microliters	*per cent*
l(−)-Aspartic acid	232	207
Glycine	198	177
l(+)-Arginine	144	129
l(+)-Lysine	138	123
l(+)-Glutamic acid	114	102
l(+)-Valine	118	105
l(−)-Phenylalanine	116	104
l(+)-Alanine	112	100
dl-Methionine	112	100
dl-Threonine	113	100
l(−)-Cysteine	117	104
l(−)-Proline	116	104
l(−)-Cysteic acid	117	104
Keto acid		
Pyruvic acid	1.5	
α-Ketoglutaric acid	2	
Oxaloacetic acid	7.5	

of 2 per cent $KMnO_4$ solution. This was allowed to stand at room temperature for 30 minutes. If the permanganate was decolorized during this interval, more was added. The solution was then directly extracted with ethyl ether. A considerable amount of MnO_2 is extracted along with the succinic acid. This does not interfere with the succinic acid determination, but, if desirable, can be readily removed by centrifugation.

Determination of Oxaloacetic Acid—Oxaloacetic acid was determined by the method of Ostern (12) as modified by Edson (13). At the end of the incubation period 0.5 ml. of 50 per cent citric acid solution was added to the incubation mixture and the acidified solution placed in the refrigerator. 0.5 ml. of aniline citrate is placed in the side arm of the Warburg vessel, while an aliquot (usually 2 ml.) of the acidified incubation mixture plus 0.5 ml. of 50 per cent citric acid solution is placed in the main compartment. The determination is carried out at 21°.

Results

Glutamic Acid Formation from α-Ketoglutaric Acid and Different Amino Acids—The percentage transamination (percentage glutamic acid formation) of different amino acids in the presence of α-ketoglutaric acid and transaminase is listed in Table II. As can been seen, the most active amino acid is *l*(−)-aspartic acid; *l*(+)-alanine and *l*(−)-cysteic acid are somewhat less active, while *l*(+)-valine shows only a slight activity. None of the remaining amino acids listed shows any appreciable activity. It is to be noted that neither of the *d*-amino acids tested shows any activity, although the corresponding *l*-amino acids are somewhat active. These results with transaminase are in agreement with those previously reported by the author for minced pigeon breast muscle (2), in which the only amino acids appreciably active in transamination with α-ketoglutaric acid are *l*(−)-aspartic acid and *l*(+)-alanine. (*l*(−)-Cysteic acid was not studied.) The results for the two amino acids differ only in the relative rates of transamination, which will be discussed in the next paper.

Braunstein (9) has recently published values for transamination of different amino acids, using minced pigeon breast muscle and purified enzyme preparations. He found that while *l*(+)-alanine was transaminated to roughly the same extent with both the minced muscle and the enzyme preparation, the amino acids *l*(+)-valine, *l*(−)-leucine, and *l*(+)-isoleucine were transaminated to only one-third the extent with the enzyme preparation. Thus with minced pigeon breast muscle Braunstein reports that these amino acids are transaminated to the extent of 17 to 21 per cent, while with the purified enzyme preparation they are transaminated to an extent of less than 7 per cent.

Aspartic Acid Formation from Oxaloacetic Acids and Different Amino Acids—The percentage transamination (percentage aspartic acid formation) of different amino acids in the presence of oxaloacetic acid and transaminase is shown in Table III. From this it can be seen that of the amino acids tested, only *l*(+)-glu-

TABLE II

Glutamic Acid Formation from α-Ketoglutaric Acid and Different Amino Acids

Each flask contains 3 ml. of transaminase solution plus 0.3 ml. of 0.2 M α-ketoglutaric acid; 1 ml. of 0.06 M *d*- or *l*-amino acid (0.12 M for *dl*-) added as indicated; substrate concentration, 0.014 M; pH 7.5; incubation time, 60 minutes; temperature 38°.

Amino acid	Glutamic acid found	Increase due to added amino acid	Transamination
	microliters	*microliters*	*per cent*
None	77		
l(−)-Aspartic acid	430	353	26.3
l(+)-Alanine	347	270	20.1
l(−)-Cysteic acid	325	248	18.5
l(+)-Valine	122	45	
l(−)-Phenylalanine	92	15	
l(+)-Arginine	92	15	
l(+)-Tryptophane	91	14	
l(−)-Cysteine	84	7	
l(+)-Lysine	84	7	
dl-Methionine	64		
l(−)-Leucine	66		
dl-Histidine	67		
β-Alanine	86	9	
Glycine	70		
Amino acids of *d* series			
d(−)-Alanine	85	8	
d(−)-Valine	90	13	

tamic acid and *l*(−)-cysteic acid are active. None of the *d*-amino acids is active. The activity of *l*(−)-cysteic acid is of considerable interest, since it reacts with both α-ketoglutaric acid (Table II) and oxaloacetic acid. Braunstein (9) has reported this amino acid to be active with pigeon breast muscle brei but inactive with puri-

fied enzyme preparations. In both instances pyruvic acid was used as the ketonic acid. The activity of cysteic acid is explained by Braunstein on the grounds that it is a dibasic amino acid and so acts like aspartic and glutamic acids. According to Braunstein the electrostatic configuration rather than the presence of specific

TABLE III

Aspartic Acid Formation from Oxaloacetic Acid and Different Amino Acids

Each flask contains 3 ml. of transaminase solution; 1 ml. of 0.06 M *d*- or *l*-amino acid (0.12 M for *dl*-) plus 0.3 ml. of 0.2 M oxaloacetic acid added as indicated; substrate concentration, 0.014 M; pH 7.5; incubation time, 60 minutes; temperature 38°.

Amino acid	CO_2 formed		Δ CO_2	Transamination
	Without oxaloacetic acid	With oxaloacetic acid		
	microliters	*microliters*	*microliters*	*per cent*
l(+)-Glutamic acid	1325	2360	+995	75
l(−)-Cysteic acid	1400	1595	+195	13.9
l(+)-Alanine	1400	1440	+40	
l(+)-Valine	1440	1478	+38	
l(−)-Phenylalanine	1415	1450	+35	
dl-Methionine	1340	1360	+20	
dl-Threonine	1450	1465	+15	
l(+)-Arginine	1730	1740	+10	
l(−)-Cysteine	1390	1350	−40	
l(−)-Proline	1390	1370	−20	
l(+)-Lysine	1655	1620	−35	
Phosphoserine	1216	1192	−24	
Amino acids of *d* series				
d(−)-Glutamic acid	1350	1360	+10	
d(−)-Valine	1500	1460	−40	
d(+)-Phenylalanine	1485	1480	−5	

carboxyl groups is the determining factor in the affinity of the dibasic amino acids. Thus in addition to cysteic acid he reports homocysteic acid and phosphoserine to be active. However, the latter does not appear to be active with oxaloacetic acid and transaminase (Table III). It is of interest to note from Tables II and III that aside from *l*(+)-alanine the only amino acids reacting

with α-ketoglutaric and oxaloacetic acids are dibasic amino acids. Of these, glutamic acid is the most active (Table III).

The optical specificity of transaminase is strikingly demonstrated from the data in Table III. Thus while $l(+)$-glutamic acid forms 995 microliters of aspartic acid in the presence of transaminase and oxaloacetic acid, $d(-)$-glutamic acid forms no significant amount.

Transamination of Glycine and Glutathione—As previously shown, glycine yields approximately 2 moles of CO_2 when treated with chloramine-T. Since alanine and glutamic acid yield but 1 mole of CO_2 under the same conditions, the decrease of CO_2 production in incubation mixtures of glycine, transaminase, and

TABLE IV

Transamination of Glycine and Glutathione with α-Keto Acids

Each flask contains 3 ml. of transaminase solution plus 1 ml. of 0.06 M glycine or glutathione; 0.3 ml. of keto acid added as indicated; substrate concentration, 0.014 M; pH 7.5; incubation time, 60 minutes; temperature 38°.

Substrates	CO_2 found	Δ CO_2
	microliters	*microliters*
Glycine	2370	
" + pyruvic acid	2390	+20
" + α-ketoglutaric acid	2430	+60
Glutathione	1115	
" + oxaloacetic acid	1085	−30

pyruvic or α-ketoglutaric acid can be employed as a measure of transamination. Such experiments are listed in Table IV. As can be seen, glycine does not undergo transamination with either pyruvic or α-ketoglutaric acid. Further evidence for this can be seen from Table II in which no glutamic acid formation occurs when glycine, α-ketoglutaric acid, and transaminase are incubated. Previous studies by the author (2) have also shown that glycine is not active with minced pigeon breast muscle.

Since glutathione is a substituted dibasic amino acid, its ability to participate in a transamination reaction was investigated. It can be seen from Table IV that there is no evidence of aspartic acid formation when glutathione is incubated with oxaloacetic acid and transaminase.

Effect of Boiled Muscle Extract on Transamination—Kritzmann (4) has reported that a thermostable cofactor prepared from muscle tissue is necessary for the action of enzyme preparations catalyzing transamination reactions in which aspartic acid partici-

TABLE V

Effect of Boiled Muscle Extract on Transamination

Each flask contains 3 ml. of transaminase solution; 1 ml. of boiled muscle extract; 0.3 ml. of 0.2 M amino acid and 0.3 ml. of 0.2 M keto acid as indicated; substrate concentration, 0.013 M; pH 7.5; temperature 38°.

Substrates	Incubation time	Found (oxaloacetic)	Δ	Transamination
Transaminase and muscle extract from pigeon breast muscle				
	min.	*microliters*	*microliters*	*per cent*
$l(-)$-Aspartic acid	15	0		
" + muscle extract	15	1.6		
" + pyruvic acid	15	1.4		
" + " + muscle extract	15	3.7		
		α-Ketoglutaric		
$l(+)$-Glutamic acid	60	98		
Muscle extract	60	33		
$l(+)$-Glutamic + pyruvic acid	15	265	167	12.5
" + "	60	476	378	25.7
" + " + muscle extract	15	280	149	11.1
" + " + " "	60	438	307	28.0
Transaminase and muscle extract from pig heart muscle				
$l(-)$-Aspartic acid	15	9.5		
" + muscle extract	15	9.2		
" + pyruvic acid	15	10.4		
" + " + muscle extract	15	7.8		
" + α-ketoglutaric acid	15	251		18.6
" + " + muscle extract	15	243		18.1

pates. In view of the fact that Reaction 3 was not catalyzed by the transaminase preparations used in this study (see Tables III, VI, and VII), the effect of concentrated muscle extract on this reaction was investigated.

Enzyme solutions prepared from pigeon breast and pig heart muscle were studied in the presence and absence of extracts from the same tissues. Transamination in these instances was followed by measuring the amount of oxaloacetic acid formed in the case of Reactions 1,*b* and 3,*a*, and the amount of α-ketoglutaric acid in Reaction 2,*a*. As can be seen from Table V, boiled muscle extract is without effect on any of the reactions studied. One must conclude from these experiments that the failure of transaminase to catalyze Reaction 3 is not due to the lack of a simple,

TABLE VI

Effect of Different Concentrations of α-Ketoglutaric Acid on Reaction 3,a

Each flask contains 3 ml. of transaminase solution plus 1 ml. of 0.06 M aspartic acid; 0.3 ml. of 0.2 M pyruvic acid and 0.3 ml. of 0.2, 0.02, and 0.002 M α-ketoglutaric acid added as indicated; incubation time, 60 minutes; pH 7.5; temperature 38°.

Substrates	CO_2 found	Δ CO_2	Transamination
	micro-liters	*micro-liters*	*per cent*
l(−)-Aspartic acid	2870		
" + pyruvic acid	2920	+50	
" + α-ketoglutaric acid (0.2 M)	2510	−360	25
" + pyruvic acid + α-ketoglutaric acid (0.2 M)	2440	−430	30
l(−)-Aspartic + α-ketoglutaric acid (0.02 M)	2860	−10	
" + pyruvic acid + α-ketoglutaric acid (0.02 M)	2910	+40	
l(−)-Aspartic + α-ketoglutaric acid (0.002 M)	2840	−30	
" + pyruvic acid + α-ketoglutaric acid (0.002 M)	2830	−40	

thermostable, extractable cofactor. It is also apparent from these data that boiled muscle extract is without effect on the rates of Reactions 2,*a* and 1,*b*.

Effect of Different Concentrations of Glutamic and α-Ketoglutaric Acids on Reaction 3—As previously pointed out, the failure of transaminase to catalyze Reaction 3 is not due to the lack of a thermostable cofactor. However, since the substrates of Reaction 3 can react with glutamic and α-ketoglutaric acids according to the Reactions 1 and 2, the question arises as to whether Reac-

tion 3 cannot be catalyzed by the addition of small quantities of these substances. Braunstein (9) has reported that this could be accomplished in the case of Reaction 3,*a* by the addition of small amounts of α-ketoglutaric acid in the presence of a purified enzyme preparation (glutamic aminopherase).

In Table VI some data are listed showing the effect of different concentrations of α-ketoglutaric acid on Reaction 3,*a*. It is seen that only when the concentration of added α-ketoglutaric acid

TABLE VII

Effect of Different Concentrations of Glutamic Acid on Reaction 3,b

Each flask contains 3 ml. of transaminase solution; 1 ml. of 0.06 M *l*(+)-alanine; 1 ml. of 0.06 M and 0.01 M *l*(+)-glutamic acid; 0.3 ml. of 0.2 M oxaloacetic acid added as indicated; incubation time, 60 minutes; pH 7.5; temperature 38°.

Substrates	CO_2 found	Δ CO_2	Transamination
	micro-liters	*micro-liters*	*per cent*
l(+)-Alanine	1435		
" + oxaloacetic acid	1410	−25	
l(+)-Glutamic acid (0.06 M)	1360		
" + oxaloacetic acid	2340	+950	72
l(+)-Alanine + oxaloacetic acid + *l*(+)-glutamic acid (0.06 M)	3700	+905*	67
l(+)-Glutamic acid (0.01 M)	207		
" + oxaloacetic acid	385	+178	86
l(+)-Alanine + oxaloacetic acid + *l*(+)-glutamic acid (0.01 M)	1810	+168†	82

* 3700 − (1360 + 1435).
† 1810 − (207 + 1435).

is high (0.2 M) is there any influence on Reaction 3,*a*. However, the increase in transamination is only 5 per cent in this instance. The failure of the lower concentrations of α-ketoglutaric acid to influence Reaction 3,*a* rules out the possibility of this substance acting as a catalyst. In Table VII some data on the effect of different concentrations of glutamic acid on Reaction 3,*b* are given. Here again there is no evidence that glutamic acid can catalytically influence the rate of Reaction 3. The lower values found in the instances where *l*(+)-alanine is present in addition to oxalo-

acetic and the different concentrations of glutamic acid are not significant.

Effect of Pyruvic and Oxaloacetic Acids on Reactions 1,a and 2,a—The effect of oxaloacetic acid on Reaction 2,*a*, and of pyruvic acid on Reaction 1,*a* is shown in Table VIII. As can be seen, the presence of equivalent amounts of pyruvic and oxaloacetic acids has no effect on the amount of aspartic acid formed by transamination, but does influence the amount of α-ketoglutaric acid formed. If Reactions 1,*a* and 2,*a* proceeded more or less independently in the presence of equal amounts of pyruvic and oxaloacetic acids, as might be expected from the fact that the aspartic acid formation is uninfluenced, then the amount of α-ketoglutaric

TABLE VIII

Effect of Oxaloacetic Acid on Reaction 2,a

Each flask contains 4.5 ml. of enzyme solution plus 1 ml. of 0.09 M *l*(+)-glutamic acid; 0.3 ml. of 0.3 M oxaloacetic acid and 0.3 ml. of 0.3 M pyruvic acid added as indicated; incubation time, 60 minutes; pH 7.5; temperature 38°.

Substrates	Aspartic acid formation	α-Ketoglutaric acid formation
	per cent	*per cent*
l(+)-Glutamic acid	0	0
" + pyruvic acid		25
" + oxaloacetic acid	63	60
" + pyruvic + oxaloacetic acid	62	72

acid formed should be additive for the two separate reactions; that is, 85 per cent. Actually, only 72 per cent is realized, indicating that in the presence of oxaloacetic acid Reaction 2,*a* is inhibited (to the extent of 50 per cent), doubtless on a competitive basis, while in the presence of pyruvic acid Reaction 1,*a* is unaffected. It can be assumed from these results that both Reactions 1,*a* and 2,*a* are being catalyzed by one and the same enzyme. The more rapid rate of Reaction 1,*a* (3 times more rapid than Reaction 2,*a*) and the greater affinity of its substrates for transaminase explain why Reaction 1,*a* is uninfluenced by pyruvic acid, while Reaction 2,*a* is inhibited by oxaloacetic acid.

Effect of Di- and Tribasic Acids—Braunstein (9) has reported

that small concentrations of dibasic acids competitively inhibit transamination. In Table IX are listed data which show the effect of di- and tribasic acids on Reaction 1,*a*. As can be seen, there is no inhibition of transamination, even though the concentration of the di- and tribasic acids is as great as that of the oxaloacetic and the glutamic acids. These results are in keeping with those found by the author (2) in pigeon breast muscle with malonic and pyrophosphoric acids, which in similar concentrations also had no effect on transamination.

TABLE IX

Effect of Di- and Tribasic Acids on Transamination

Each flask contains 3 ml. of transaminase solution plus 1 ml. of 0.06 M *l*(+)-glutamic acid; 0.3 ml. of 0.2 M oxaloacetic acid and 0.3 ml. of 0.2 M di- and tribasic acids added as indicated; substrate concentration, 0.013 M; di- and tribasic acid concentration, 0.013 M; pH 7.5; temperature 38°; incubation time, 30 minutes.

Substrates	CO_2 found	Δ CO_2	Transamination
	micro-liters	*micro-liters*	*per cent*
l(+)-Glutamic acid	1385		
" + oxaloacetic acid	2385	+1000	72
" + " + succinic acid	2410	+1025	74
" + " + malonic "	2410	+1025	74
" + " + pyrophosphoric acid	2395	+1010	73
l(+)-Glutamic + oxaloacetic + citric acid	2390	+1005	72

DISCUSSION

Transamination with Pigeon Breast Muscle and Transaminase—It is apparent from the data presented here that of the many amino acids tested only *l*(+)-alanine, *l*(+)-glutamic, *l*(−)-aspartic, and *l*(−)-cysteic acids are active in transamination with transaminase. Aside from *l*(−)-cysteic acid, which was not investigated in a previous study with pigeon breast muscle by the author (2), the same amino acids are active in both cases. This means that the transaminase preparations have lost none of their transamination activity as far as substrate activation is concerned. The comparison of the rates of reaction with the two systems will be pre-

sented in the following paper. Whether or not the transamination activity resides in a single enzyme which activates all the amino acids mentioned above is not apparent from these experiments. However, the data of Table VIII indicate that there is a competitive inhibition for the substrates of Reactions 1,*a* and 2,*a*, suggesting that the same enzyme catalyzes both these reactions.

Substrates Active in Transamination—In addition to Reactions 1 and 2, it is apparent from this study that transaminase catalyzes two more reactions.

$$\text{(4)}\quad l(-)\text{-Cysteic acid} + \alpha\text{-ketoglutaric acid} \underset{b}{\overset{a}{\rightleftharpoons}} l(+)\text{-glutamic acid} + \beta\text{-sulfopyruvic acid}$$

$$\text{(5)}\quad l(-)\text{-Cysteic acid} + \text{oxaloacetic acid} \underset{b}{\overset{a}{\rightleftharpoons}} l(-)\text{-aspartic acid} + \beta\text{-sulfopyruvic acid}$$

Experimental evidence for Reactions 4,*b* and 5,*b* is not as yet available.

In considering the chemical nature of the substrates active in transamination, it is seen that, with the exception of pyruvic acid and $l(+)$-alanine, all the substrates are dibasic acids. It should also be noted that in the instances where the monobasic acids participate in a transamination reaction (Reaction 2) they are active only when the dibasic acid is α-ketoglutaric or glutamic acid. On the other hand, when both substrates are dibasic acids (as in Reactions 1, 4, and 5), glutamic acid is not essential (Reaction 4).

Transamination in Tissues Other Than Muscle—The question as to whether transamination is qualitatively and quantitatively the same in different tissues is at present under investigation. It appears that more amino acids are transaminated in liver and kidney (20) than in pigeon breast muscle. Whether these tissues yield different transaminases is being studied at present.

Results of Braunstein and Coworkers—Braunstein, who with his coworkers, has done the pioneer work on transamination, has recently reviewed this subject (9). The results of these workers differ in the main from those reported by the author in that they consider transamination to be a general reaction in which many

amino acids are involved, while it is the view of the writer that the reaction is a limited one, in muscle, at any rate, as regards substrate specificity. In addition, the experimental evidence offered by Braunstein and his coworkers in proof of (1) the existence of two separate enzyme systems, one for glutamic and the other for aspartic acids, the latter requiring a cofactor, (2) the catalytic effect of α-ketoglutaric acid on Reaction 3, and (3) the inhibitory action of dibasic acids on transamination is not supported by the experiments in this study. These differences in results are undoubtedly due to the different analytical methods and techniques employed.

SUMMARY

1. By using the method of Kritzmann, it was found that both pigeon breast and pig heart muscle yield an enzyme (or enzymes), here called transaminase, which catalyzes the reactions

$$\text{(1)}\quad l(+)\text{-Glutamic acid} + \text{oxaloacetic acid} \underset{b}{\overset{a}{\rightleftharpoons}} \alpha\text{-ketoglutaric acid} + l(-)\text{-aspartic acid}$$

$$\text{(2)}\quad l(+)\text{-Glutamic acid} + \text{pyruvic acid} \underset{b}{\overset{a}{\rightleftharpoons}} \alpha\text{-ketoglutaric acid} + l(+)\text{-alanine}$$

$$\text{(4)}\quad l(-)\text{-Cysteic acid} + \alpha\text{-ketoglutaric acid} \underset{b}{\overset{a}{\rightleftharpoons}} l(+)\text{-glutamic acid} + \beta\text{-sulfopyruvic acid}$$

$$\text{(5)}\quad l(-)\text{-Cysteic acid} + \text{oxaloacetic acid} \underset{b}{\overset{a}{\rightleftharpoons}} l(-)\text{-aspartic acid} + \beta\text{-sulfopyruvic acid}$$

(Experimental evidence for Reactions 4, *b* and 5, *b* is not available to date.)

but not the reaction,

$$\text{(3)}\quad l(-)\text{-Aspartic acid} + \text{pyruvic acid} \underset{b}{\overset{a}{\rightleftharpoons}} \text{oxaloacetic acid} + l(+)\text{-alanine}$$

2. Boiled muscle extract is without influence on any of the above reactions. Further, Reaction 3 is not influenced by the presence of catalytic amounts of either α-ketoglutaric or $l(+)$-glutamic acid.

3. Amino acids of the *l* series only are active with transaminase.
4. Di- and tribasic acids do not inhibit transaminase.
5. Glutathione is not active in transamination.
6. A new manometric method is described for measuring the formation and disappearance of aspartic acid. It is based on the fact that aspartic acid yields 2 moles of CO_2 with chloramine-T, while most other amino acids yield but 1. This method is particularly adapted for studying reactions with transaminase in which oxaloacetic or aspartic acid participates.

The author wishes to express his thanks to Professor C. N. H. Long for his interest in this work.

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KINETICS OF TRANSAMINASE ACTIVITY

By PHILIP P. COHEN*

(*From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*)

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In the preceding paper (1) the activity of transaminase in catalyzing the transamination of various α-amino and α-keto acids was investigated. In addition, the influence of cofactors and other compounds was studied. The experiments to be described in this paper deal with (*a*) some physical constants of transaminase and (*b*) kinetic studies of the reactions,

$$\text{(1)}\quad l(+)\text{-Glutamic acid} + \text{oxaloacetic acid} \underset{b}{\overset{a}{\rightleftharpoons}} \alpha\text{-ketoglutaric acid} + l(-)\text{-aspartic acid}$$

$$\text{(2)}\quad l(+)\text{-Glutamic acid} + \text{pyruvic acid} \underset{b}{\overset{a}{\rightleftharpoons}} \alpha\text{-ketoglutaric acid} + l(+)\text{-alanine}$$

$$\text{(3)}\quad l(-)\text{-Aspartic acid} + \text{pyruvic acid} \underset{b}{\overset{a}{\rightleftharpoons}} \text{oxaloacetic acid} + l(+)\text{-alanine}$$

Methods and Procedures

The experimental and analytical procedures employed in this study were the same as those described in the previous paper (1). In addition to the determinations of glutamic, aspartic, and α-ketoglutaric acids, pyruvic acid was determined by the carboxylase[1] method of Westerkamp (2). At the end of the incubation period 1 ml. of 3 M acetic acid-acetate buffer, pH 4.5, was added to the incubation mixtures. The solutions were then transferred to graduated

* National Research Council Fellow in Medical Sciences.

[1] The author wishes to express his thanks to Dr. Joseph L. Melnick for a supply of dried brewers' yeast.

tubes and an aliquot taken for analysis. The determination of pyruvic acid in the presence of α-ketoglutaric acid is possible because of the slow rate at which the latter is decarboxylated. Thus in the presence of mixtures of the two, practically all the pyruvic acid is decarboxylated during the first 15 minutes of contact with the carboxylase, while only a small amount of α-ketoglutaric acid reacts.

Unless otherwise indicated in the different experiments, the source of transaminase was pig heart muscle.

Results

Kinetics of Transamination

Velocity Constants of Reactions 1,a and 1,b—The determination of the true velocity constants of Reactions 1,*a* and 1,*b* is difficult owing to the relatively rapid rates of the reverse reactions. However, some measurements were made at short incubation periods in an attempt to confine the reactions to their initial rates and so to minimize the rates of the reverse reactions. In Table I are presented velocity constants for Reactions 1,*a* and 1,*b* calculated on the basis of a monomolecular and a bimolecular reaction. Calculated on the basis of a monomolecular reaction, the initial rates appear to be constant during the first 10 minutes, but start falling off rapidly as equilibrium is approached. The values calculated for a bimolecular reaction appear to be somewhat less variable with time.

The equilibrium constant, calculated from the velocity constants, for Reaction 1 (Ka/Kb = equilibrium constant) varies from 3.14 to 3.84, and from 3.69 to 5.10, as determined from the monomolecular velocity constants in the former and the bimolecular in the latter.

Rates of Reaction Product Formation and Substrate Disappearance in Reaction 1—With the demonstration that Reaction 1 is catalyzed by transaminase preparations (1), the rate of reaction product formation and substrate disappearance was investigated. This was done by measuring simultaneously the rates of α-ketoglutaric and aspartic acid formation in the forward reaction (Reaction 1,*a*) and the rates of glutamic acid formation and aspartic acid disappearance in the reverse reaction (Reaction 1,*b*). As can be seen from Table II, the rates of transamination are the

same whether α-ketoglutaric or aspartic acid formation is measured in Reaction 1,*a*, or glutamic acid formation or aspartic acid disappearance in Reaction 1,*b*. These data prove definitely that Reaction 1 proceeds as indicated and that an accurate determination of the formation or disappearance of any one of the four components involved will serve as a measure of transamination provided equilibrium has been established. Further, the α-ketoglutaric and glutamic acid values prove the validity of the method

TABLE I

Velocity Constants of Reactions 1,a and 1,b

Initial substrate concentration, 0.014 M; pH 7.5; temperature 38°.

Time	Transaminated	$K = \frac{1}{t} \log \frac{a}{a-x}$ (monomolecular)	$K = \frac{x}{t(a-x)a}$ (bimolecular)
Reaction 1,*a* (initial glutamic and oxaloacetic acid concentration, 60 micromoles)			
min.	*micromoles*		
5	18.4	0.031	0.00147
10	31.5	0.032	0.00184
15	35.3	0.026	0.00159
30	43.3	0.018	0.00144
Reaction 1,*b* (initial α-ketoglutaric and aspartic acid concentration, 60 micromoles)			
5	6.4	0.0090	0.000398
10	11.8	0.0097	0.000408
15	15.0	0.0083	0.000370
30	16.8	0.0047	0.000282

used for measuring aspartic acid formation and disappearance (1).

Rates and Position of Equilibrium of Reactions 1, 2, and 3 with Transaminase Preparations from Pigeon Breast Muscle and Pig Heart Muscle—The rates of Reactions 1 and 2 are represented graphically in Figs. 1 and 2. As can be seen, the initial rate for Reaction 1,*a* is very rapid, approximately 50 per cent of the added substrate being transformed in the first 10 minutes. While Reaction 1,*b* is much slower than Reaction 1,*a*, the initial rate is also comparatively rapid. On the other hand, Reactions 2,*a* and 2,*b* proceed at a slow rate, with comparatively slow initial rates.

From Tables III and IV it would appear that the initial rates for Reactions 1 and 2 are somewhat faster in the presence of pigeon

TABLE II

Rate of Aspartic Acid Formation and Disappearance, and Glutamic and α-Ketoglutaric Acid Formation in Reaction 1

Each flask contains 4.5 ml. of transaminase solution; 1 ml. of 0.09 M amino acid and 0.45 ml. of 0.2 M α-keto acid added as indicated; phosphate buffer added to make substrate concentration 0.014 M; pH 7.5; incubation temperature 38°.

Aspartic acid formation	Incubation time	Found, CO_2	Δ	Transamination
	min.	*micro-liters*	*micro-liters*	*per cent*
$l(+)$-Glutamic acid	60	1975		
" + oxaloacetic acid	15	3000	+1025	52
" + " "	30	3420	+1445	73
" + " "	60	3480	+1505	76
		α-Ketoglutaric acid		
α-Ketoglutaric acid formation				
$l(+)$-Glutamic acid	60	20		
" + oxaloacetic acid	15	1080	+1060	53
" + " "	30	1336	+1316	66
" + " "	60	1440	+1420	72
		CO_2		
Aspartic acid disappearance				
$l(-)$-Aspartic acid	60	4320		
" + α-ketoglutaric acid	15	3860	−460	21
" + " "	30	3750	−570	26
" + " "	60	3690	−630	29
		Glutamic acid		
Glutamic acid formation				
α-Ketoglutaric acid	60	52		
" + $l(-)$-aspartic acid	15	422	+370	18
" + " "	30	558	+506	25
" + " "	60	592	+540	26

breast muscle transaminase than that from pig heart muscle. As would be expected, the respective points of equilibrium are the

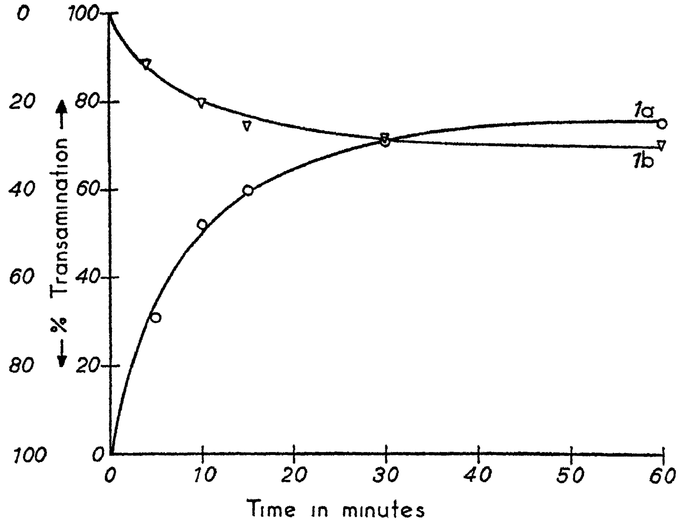

FIG. 1. Rate of Reaction 1. The ordinate to the left for Reaction 1,*b* represents the percentage aspartic acid disappearance; the ordinate to the right for Reaction 1,*a* represents aspartic acid formation; both calculated as percentage transamination; substrate concentration, 0.014 M; temperature, 38°; pH 7.5.

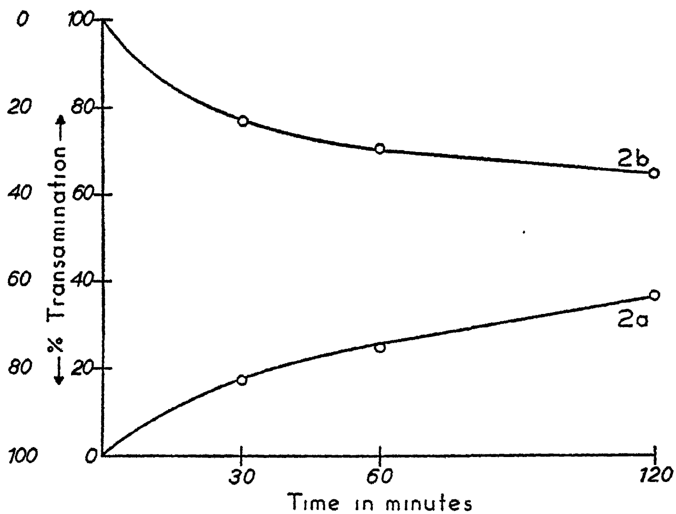

FIG. 2. Rate of Reaction 2. The ordinate to the left for Reaction 2,*b* represents the percentage glutamic acid formation; the ordinate to the right for Reaction 2,*a* represents the percentage α-ketoglutaric acid formation; both calculated as percentage transamination. Substrate concentration, 0.014 M; temperature, 38°; pH 7.5.

TABLE III

Rate of Transamination with Transaminase Prepared from Pigeon Breast Muscle in Reactions 1, 2, and 3

Each flask contains 3 ml. of transaminase solution; 1 ml. of 0.06 M amino acid and 0.3 ml. of 0.2 M α-keto acid added as indicated. Substrate concentration, 0.014 M; incubation temperature 38°; pH 7.5.

	Incubation time	Found, CO_2	Δ	Transamination
	min.	*micro-liters*	*micro-liters*	*per cent*
Reactions 1,*a* and 1,*b*				
l(+)-Glutamic acid	60	1310		
" + oxaloacetic acid	15	2100	+790	60
" + " "	60	2240	+930	71
l(−)-Aspartic acid	60	2850		
" + α-ketoglutaric acid	15	2520	−330	23.1
" + " "	60	2380	−470	33
		Pyruvic acid		
Reactions 2,*a* and 2,*b*				
Pyruvic acid	60	1325		
" + *l*(+)-glutamic acid	15	1143	−182	13.7
" + " "	60	985	−340	25.7
		α-Ketoglutaric acid		
α-Ketoglutaric acid	60	1400		
" + *l*(+)-alanine	15	1196	−204	14.6
" + "	60	1000	−400	28.6
		CO_2		
Reactions 3,*a* and 3,*b*				
l(−)-Aspartic acid	60	2850		
" + pyruvic acid	15	2830	−20	
" + " "	60	2900	+50	
l(+)-Alanine	60	1440		
" + oxaloacetic acid	15	1420	−20	
" + " "	60	1440	0	

same with the two transaminase preparations. It is apparent that Reaction 3 is not catalyzed by transaminase from either source.

TABLE IV

Rate of Transamination with Transaminase Prepared from Pig Heart Muscle in Reactions 1, 2, and 3

Each flask contains 3 ml. of transaminase solution; 1 ml. of 0.06 M amino acid and 0.3 ml. of 0.2 M α-keto acid added as indicated; substrate concentration, 0.014 M; pH 7.5; temperature 38°.

	Incubation time	Found, CO_2	Δ	Transamination
	min.	*microliters*	*microliters*	*per cent*
Reactions 1,*a* and 1,*b*				
l(+)-Glutamic acid	60	1270		
" + oxaloacetic acid	15	1820	+550	43.3
" + " "	60	2210	+940	74
l(−)-Aspartic acid	60	2725		
" + α-ketoglutaric acid	15	2440	−285	20.9
" + " "	60	2260	−465	34
		α-Ketoglutaric acid		
Reactions 2,*a* and 2,*b*				
l(+)-Glutamic acid	60	18		
" + pyruvic acid	15	122	+104	8.2
" + " "	60	276	+258	20.3
		Glutamic acid		
α-Ketoglutaric acid	60	76		
" + *l*(+)-alanine	15	212	+136	10.1
" + "	60	346	+270	20.1
		CO_2		
Reactions 3,*a* and 3,*b*				
l(−)-Aspartic acid	60	2725		
" + pyruvic acid	15	2780	+55	
" + " "	60	2710	−15	
l(+)-Alanine	60	1465		
" + oxaloacetic acid	15	1480	+15	
" + " "	60	1460	−5	

Kritzmann (3) has reported that enzyme preparations from pigeon breast muscle catalyze Reaction 2 at a rapid rate, equilibrium being attained in about 60 minutes when 50 per cent of the

added substrates has reacted. According to this, Reaction 2 attains equilibrium in the same period of time with the enzyme preparation as with pigeon breast muscle. Kritzmann did not study Reaction 1 in either pigeon breast muscle or with transaminase preparations. From a previous study made by the author (4) of Reactions 1 and 2 in pigeon breast muscle it appeared that both reactions came to equilibrium when 50 per cent of the added substrates had reacted, Reaction 1 attaining equilibrium more rapidly than Reaction 2. This would imply that Reaction 1 comes to different positions of equilibrium with pigeon breast muscle and transaminase. However, this difference can be explained by the fact that oxaloacetic acid undergoes a number of rapid reactions when added to pigeon breast muscle under anaerobic (and aerobic) conditions. The chief among these are

(*a*) Oxaloacetic acid → pyruvic acid + CO_2 (5)*
(*b*) Oxaloacetic acid → malic acid → fumaric acid → succinic acid (6)
(*c*) Oxaloacetic acid + pyruvic acid → malic acid + acetic acid + CO_2 (7)
(*d*) Oxaloacetic acid + pyruvic acid → citric acid (8)

The effect of these rapid side reactions would be such as to slow down the rate of Reaction 1,*a*, while increasing the rate of Reaction 1,*b*. The net result of this would be that seen in pigeon breast muscle, where, compared with the transaminase preparation, Reaction 1,*a* is slower, while Reaction 1,*b* is faster. This would give the impression of a different equilibrium point for the same reaction. The effect of different concentrations of oxaloacetic acid on the percentage transamination of glutamic acid can be seen from Fig. 3. Thus when the concentration of both glutamic and oxaloacetic acids is 0.014 M, the percentage transamination is 70, but, when the concentration of oxaloacetic acid is halved, the percentage transamination is decreased to 52. The latter value is approximately that found with pigeon breast muscle when glutamic and oxaloacetic acids are added in equimolecular concentrations. The rate of oxaloacetic acid disappearance by the reactions listed above is such that the concentration of added oxaloacetic acid would be halved in a very short time. In support of this are the observations by Szent-Györgyi and Straub (5) who

* The figures following the reactions, refer to bibliographic reference numbers.

reported that 7 per cent of the added oxaloacetic acid disappeared by Reaction *a* alone in 10 minutes, while Szent-Györgyi and Banga (9) found that of 8.6 mg. of added oxaloacetic acid 8 mg. disappeared in the first 10 minutes.

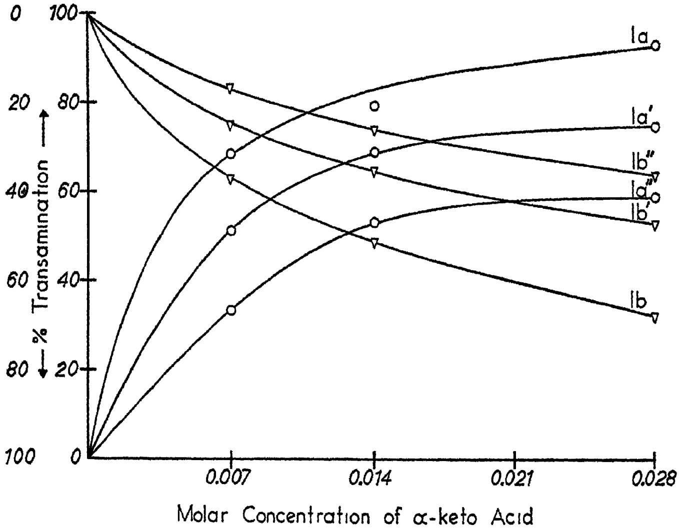

FIG. 3. Effect of different substrate concentrations on transamination in Reactions 1,*a* and 1,*b*. Glutamic acid concentrations for Reactions 1, *a* 1, *a'*, and 1, *a''* are 0.007 M, 0.014 M, and 0.028 M, respectively; oxaloacetic acid concentration indicated on the abscissa. Aspartic acid concentrations for Reactions 1, *b*, 1, *b'*, and 1, *b''* are 0.007 M, 0.014 M, and 0.028 M, respectively; α-ketoglutaric acid concentration indicated on the abscissa. The ordinate on the left for Reactions 1, *b*, 1, *b'*, 1, *b''* represents the percentage aspartic acid disappearance, the ordinate on the right for Reactions 1, *a*, 1, *a'*, 1, *a''* represents percentage aspartic acid formation; both calculated as percentage transamination. Temperature, 38°; pH 7.5; incubation time, 60 minutes.

The higher rates of transamination for Reaction 1,*b* in pigeon breast muscle as compared with transaminase preparations is explainable by the rapid removal of formed oxaloacetic acid by the reactions listed above. This would not only serve to increase the rate of Reaction 1,*b*, but would also decrease the rate of the reverse reaction, No. 1,*a*.

In pigeon breast muscle, Reaction 2 rapidly approaches a steady state when approximately 50 per cent of the substrates has reacted (4). In the case of transaminase, however, the reaction is considerably slower in attaining equilibrium (Tables III and IV; Fig. 2). As shown in the preceding paper (1), boiled muscle extract is without influence on Reaction 2. However, this does not rule out the possibility of a thermolabile cofactor for this reaction.

A comparison of the $Q_{\text{transamination}}$ values,

$$\left(Q_{\text{transamination}} = \frac{\text{microliters substrate transaminated}}{\text{mg. dry weight} \times \text{hour}}\right)$$

of pigeon breast muscle and transaminase is of interest. In a previous study (4) it was found that the $Q_{\text{transamination}}$ values of pigeon breast muscle as measured in Reactions 1,*a* and 2,*a* are of the order of 44 and 39, respectively, calculated on the basis of initial rates. With the same reactions the $Q_{\text{transamination}}$ values of transaminase are 1668 and 296, respectively. From this it would appear that with purification the activity of the enzyme as measured in Reactions 1,*a* and 2,*a* is increased 38-fold and 7.6-fold, respectively, a ratio of 5:1. This would suggest that the two reactions are catalyzed by different enzyme systems. However, it must be kept in mind that these ratios are based on the assumption that the $Q_{\text{transamination}}$ value for pigeon breast muscle as measured in Reaction 1,*a* represents the optimum value. It is certain that the $Q_{\text{transamination}}$ value of 44 is too low. Whether it is 5 times too low is impossible to say, but in view of the rapidity of the side reactions and the difficulty of measuring optimum rates at very short incubation periods a $Q_{\text{transamination}}$ value of around 200 is more probable.

It is apparent from the above $Q_{\text{transamination}}$ values that there is about an 8-fold increase in activity with purification (this is on the assumption that the 38-fold increase as measured in Reaction 1,*a* is incorrect according to the above discussion). Kritzmann (3) has reported a 12-fold increase in activity for similar preparations, as measured in Reaction 2. However, the transamination values for pigeon breast muscle used as a basis for activity comparison are very low. Calculated on a $Q_{\text{transamination}}$ basis, she finds a value of 8.9. This is about one-fourth the value reported in other publications from that laboratory.

Reaction 3 is not catalyzed by any of the transaminase preparations tested so far. Direct study of this reaction in pigeon breast muscle is difficult owing to the lack of suitable specific micromethods. Indirect studies by the author (4) have shown that the reaction does not take place to any measurable extent in pigeon breast muscle. On the other hand, Braunstein (10) has reported that Reaction 3 occurs not only in pigeon breast muscle, but also with purified enzyme preparations. According to Braunstein the enzyme preparations from pig heart and pigeon breast muscle are inactive with aspartic acid owing to the lack of a cofactor. However, in the previous paper (1) it was shown that boiled muscle extracts were without influence on any transamination reactions.

Effect of Substrate Concentration; Michaelis Constant for Transaminase—The effect of varying the concentration of glutamic acid with respect to oxaloacetic acid on transamination in Reactions 1,*a* and 1,*b* is shown in Fig. 3. If both substrates are kept at equal concentrations, the *amount* of substrate transaminated is increased with increasing concentration of substrate, but the *percentage* of substrate transaminated is decreased. With a given concentration of glutamic or aspartic acid, increasing the relative concentrations of oxaloacetic and α-ketoglutaric acids, respectively, results in an increase in the *percentage* transamination of the amino acids. From Fig. 3 it can also be seen that at substrate concentrations of 0.007 M and 0.014 M Reactions 1,*a* and 1,*b* approach equilibrium at approximately the same rate, while at a higher concentration, 0.028 M, the equilibrium is approached at a slightly slower rate.

In Fig. 4, the microliters of aspartic acid formed with varying concentrations of substrate (the concentrations of glutamic and oxaloacetic acids are equal) is shown. From this curve, the Michaelis constant, K_m, can be calculated for transaminase as indicated. The value of $-\log S$ at the point of half the maximum rate of aspartic acid formation is -1.86. This corresponds to a Michaelis constant, K_m, of 0.0138 M.

Effect of Temperature—The effect of temperature on transaminase activity measured in Reaction 1,*a* is shown in Fig. 5. The optimum temperature appears to be 40°, beyond which the rate of the reaction slowly decreases. The crude enzyme (moist precipitate) is apparently less thermolabile, since it can withstand heating to 70° for 20 minutes, as first pointed out by Kritzmann (3). Be-

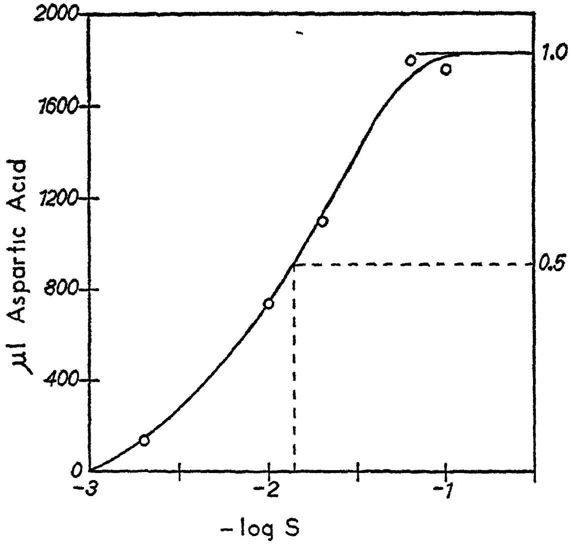

FIG. 4. Effect of substrate concentration on transaminase activity; Michaelis constant. The abscissa represents the negative logarithm of the substrate concentration (glutamic and oxaloacetic acids); the left ordinate represents microliters of aspartic acid formed; the right ordinate, points of maximum and half maximum rates. Temperature, 38°, pH 7.5; incubation time, 15 minutes. K_m calculated from parameters as indicated.

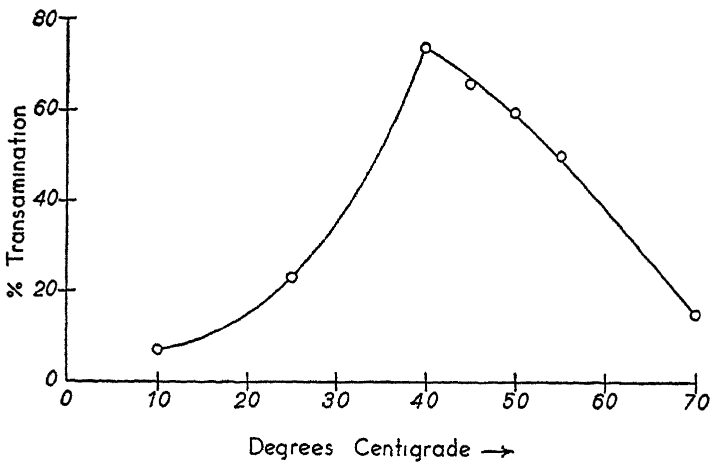

FIG. 5. Effect of temperature (Reaction 1, *a*). Substrate concentration, 0.014 M; incubation time, 60 minutes; pH 7.5. The ordinate represents the percentage of aspartic acid formation calculated as percentage transamination.

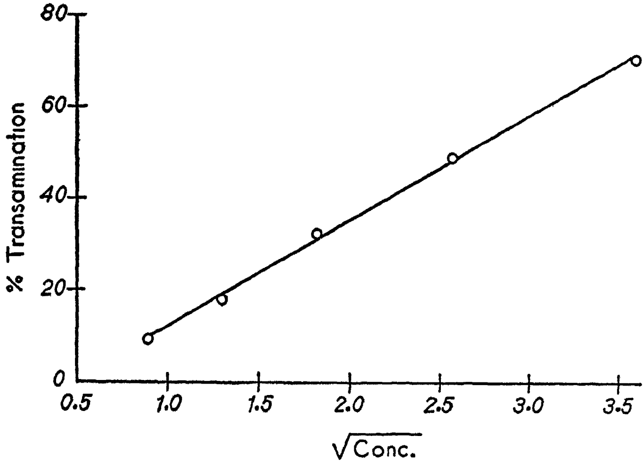

FIG. 6. Effect of enzyme concentration on Reaction 1, *a*. The abscissa represents the square root of the mg. of protein present in 3 ml. of the different enzyme solutions; the ordinates represent the percentage aspartic acid formation calculated as percentage transamination. Substrate concentration, 0.014 M; incubation time, 30 minutes; temperature, 38°; pH 7.5.

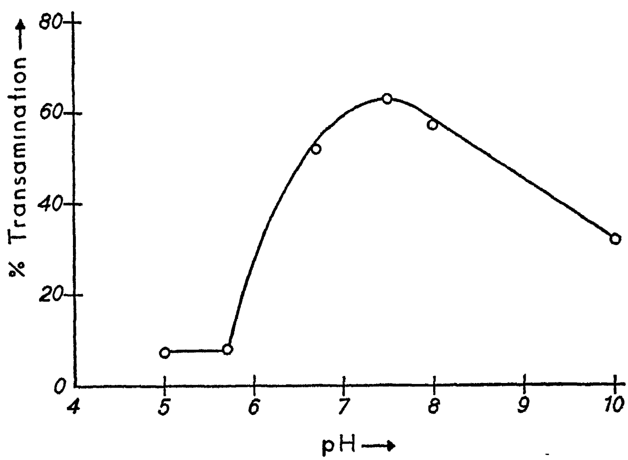

FIG. 7. pH-activity curve for transaminase. The abscissa represents the pH of incubation mixtures; the ordinate represents the percentage aspartic acid formation calculated as percentage transamination. Substrate concentration, 0.014 M; incubation time, 30 minutes; temperature, 38°.

tween 20–40°, the rate of transamination is approximately doubled with each 10° rise in temperature.

Effect of Enzyme Concentration—The effect of enzyme concentration on the percentage transamination in Reaction 1,*a* is shown in Fig. 6. It appears that the percentage transamination is proportional to the square root of the enzyme concentration. This is in agreement with the so called law of Schütz (11) which states that the velocity of a reaction is proportional to the square root of the concentration of the enzyme. However, since the percentage transamination values represent rates measured after the reaction has attained equilibrium, it is obvious that they do not represent true reaction velocities. Thus the relationship seen in Fig. 6 may not hold when absolute velocities for Reaction 1,*a* are plotted against enzyme concentration.

Effect of pH—The effect of pH on transaminase activity as measured by Reaction 1,*a* is shown in Fig. 7. The optimum pH for Reaction 1,*a* lies at about 7.5. Reaction 2,*a* has a pH optimum in the same region. Kritzmann (3) has also reported an optimum pH of 7.5 for Reaction 2.

DISCUSSION

Identity of Transaminase Preparations Catalyzing Reactions 1 and 2—The question as to whether Reactions 1 and 2 are catalyzed by one and the same enzyme is not conclusively settled from this study. However, from the experiments in the preceding paper (1) it was seen that the substrates of Reactions 1,*a* and 2,*a* compete for the same enzyme. It is seen from the experiments in this paper that both reactions have approximately the same pH optima, and further, that the transaminase preparations from two different sources, pigeon breast and pig heart muscle, behave identically. The one observation in favor of more than one enzyme is the difference in $Q_{\text{transamination}}$ values with purification as measured in Reactions 1,*a* and 2,*a*. However, as previously discussed, this difference may well be more apparent than real. The above facts taken together support the idea that one and the same enzyme catalyzes Reactions 1 and 2. The chief substrates for this enzyme are glutamic and oxaloacetic acids, on the one hand, and α-ketoglutaric and aspartic acids, on the other. Pyruvic acid and alanine have a limited affinity for the enzyme and therefore Reac-

tion 2 proceeds at a considerably slower rate than Reaction 1. If only one of the pair of substrates is a dicarboxylic acid, it must be either glutamic or α-ketoglutaric acid. Thus Reaction 3 is not catalyzed by transaminase (Tables III and IV). On the other hand, if the pair of substrates includes an α-amino dibasic acid and an α-keto dibasic acid, then glutamic acid is not required (1). This must mean that transaminase is chiefly concerned with the catalysis of reactions in which both substrates are dibasic acids (one an α-amino and the other an α-keto acid).

Rôle of Transamination in Intermediary Metabolism—The rapid rate of Reaction 1 indicates that $l(+)$-glutamic and oxaloacetic acids, on the one hand, and $l(-)$-aspartic and α-ketoglutaric acids, on the other, represent the chief substrates for transaminase. The $Q_{\text{transamination}}$ value for this reaction in pigeon breast muscle is probably of the order of 200, which is several times greater than the Q_{O_2} value for that tissue. The substrates of Reaction 1 are also known to play important rôles in the intermediary metabolism of muscle. Thus not only are $l(-)$-aspartic acid and $l(+)$-glutamic acid dehydrogenated by muscle (12–16) but they also act as respiratory catalysts (17). Glutamic acid, in addition, has been shown to act as a hydrogen carrier not only through virtue of the fact that its dehydrogenase can function with both di- and triphosphopyridine nucleotides (15, 18), and so can couple with other di- and triphosphopyridine nucleotide-catalyzed systems (18, 19), but also by a dismutation reaction involving α-ketoglutaric acid and NH_3 (20). The reactions of pyruvic acid and the key rôles of oxaloacetic and α-ketoglutaric acids as respiratory mediators in the Szent-Györgyi-Krebs cycle have recently been elucidated by Krebs (21, 22).

It thus becomes apparent that transamination is not only an extremely rapid reaction in pigeon breast muscle with $Q_{\text{transamination}}$ values several times greater than the Q_{O_2} values, but that it is also concerned with those very substances which have been shown to play key rôles in the intermediary metabolism of muscle. This would indicate that the rôle of transamination is one of coupling or providing a common source or pathway for these important metabolites. In view of the rapid rate of transamination and its independence of aerobic conditions the importance of this reaction in making available such substances as α-ketoglutaric and oxalo-

acetic acids for muscle metabolism becomes apparent. While this may not be the only rôle which transamination plays, it would appear to be the chief rôle in muscle.

SUMMARY

1. In the presence of transaminase, Reaction 1 has an equilibrium constant (Ka/Kb = equilibrium constant) of approximately 3.5, and Reaction 2 of approximately 1. The different equilibrium constant observed in pigeon breast muscle for Reaction 1 is discussed and explained. Reaction 2 has the same equilibrium constant as in pigeon breast muscle.

2. Reactions 1 and 2 are probably catalyzed by the same enzyme (transaminase).

3. The $Q_{\text{transamination}}$ values (calculated on the basis of initial rates) of transaminase for Reactions 1,*a* and 2,*a* are 1668 and 296, respectively, which represent an activity increase of 38- and 7.6-fold when compared with pigeon breast muscle. However, the 38-fold activity increase for Reaction 1,*a* is probably incorrect owing to the difficulty of measuring the optimum rate of this reaction in pigeon breast muscle.

4. Transaminase has an optimum activity at 40° and at pH 7.5, with a Michaelis constant for Reaction 1,*a* of 0.0138 M.

5. From this and previous studies by the author it appears that the chief substrates for transaminase from pigeon breast and pig heart muscle are a dibasic α-amino *plus* a dibasic α-keto acid.

6. The rôle of transamination in muscle metabolism is discussed.

The author wishes to express his thanks to Professor C. N. H. Long for his interest in this work. The author is also indebted to Mr. G. Leverne Hekhuis for technical assistance in certain phases of this study.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LXII. STUDIES ON MYCOLIC ACID*

By ALEX LESUK† AND R. J. ANDERSON

(*From the Department of Chemistry, Yale University, New Haven*)

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Mycolic acid is the principal ether-soluble constituent obtained on saponification of the purified wax (1), of the soft wax (2), and of the wax obtained in the purification of the phosphatide (3) of the human tubercle bacillus and it is the only substance that we have been able to isolate from the bacillus that possesses the property of acid fastness. It has been shown by Sabin and collaborators (4) that mycolic acid stimulates the proliferation of connective tissue cells when it is introduced subcutaneously into normal animals, but it does not show any specific stimulating effect on monocytes or epithelioid cells such as is exhibited by the phosphatide (5) or by phthioic acid (6).

In view of the abundance of mycolic acid in the tubercle bacillus we may assume that it plays an important rôle in the life of the organism but its actual function is as yet unknown. Chemically, mycolic acid is very inert and it is extremely stable against both acids and alkali; hence it may be one of the chief substances which protect the bacillus against destructive attacks by cells or enzymes of the host.

In earlier work reported from this laboratory (7) it has been established that mycolic acid is a saturated compound of high

* The data are taken from the dissertation submitted by Alex Lesuk to the Faculty of the Graduate School, Yale University, 1939, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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† Holder of a National Tuberculosis Association Fellowship at Yale University, 1937–39.

molecular weight and that it contains hydroxyl and methoxyl groups. The average equivalent weight determined by titration of several of the purest samples of the acid was 1284 but individual preparations varied from 1270 to 1316. The simplest formula calculated from combustion analysis is $C_{88}H_{172}O_4$ or $C_{88}H_{176}O_4$. However, since mycolic acid does not crystallize, we have no adequate criteria of homogeneity. The acid as isolated may represent a mixture of two or more acids which possess very similar properties and by reason of similar solubilities cannot be separated by present methods of purification. In fact, some results reported in the experimental part indicate that mycolic acid is not homogeneous. New methods of purification must be developed before any definitely homogeneous mycolic acid can be isolated.

It has been found in earlier experiments that mycolic acid decomposes when it is heated to 250–300° under reduced pressure and that normal hexacosanoic acid, $C_{26}H_{52}O_2$, distils off, leaving a nearly colorless, unsaturated, non-volatile residue (7). This reaction might be explained on the theory that mycolic acid is a coordination compound, but as will be shown in the experimental part such a theory is untenable because normycolic acid does not yield hexacosanoic acid when heated under reduced pressure.

It has been found in the present investigation that when mycolic acid is refluxed with hydriodic acid as in the usual Zeisel determination only the methoxyl group is attacked, resulting in the formation of monoiodohydroxynormycolic acid. On reduction with zinc dust and acetic acid this compound is converted into hydroxynormycolic acid and this substance on pyrolysis yields normal hexacosanoic acid and a neutral non-volatile residue.

It has also been found that when mycolic acid is refluxed with more concentrated hydriodic acid both the methoxyl and the hydroxyl groups are eliminated with the formation of diiodonormycolic acid. This compound on reduction yields an acid which contains neither methoxyl nor hydroxyl and may be designated normycolic acid. This acid is optically inactive and when subjected to pyrolysis under reduced pressure does not yield hexacosanoic acid but is apparently decarboxylated. It is evident, therefore, that mycolic acid is not a coordination compound and that the hydroxyl group is essential for the pyrolytic cleavage to occur with the liberation of hexacosanoic acid.

Normycolic acid is not the only product obtained on reduction of the iodo compound. It is possible to separate from the reduction product by means of the slightly soluble potassium salt a monohydroxymonocarboxylic acid which possesses a higher molecular weight than the original mycolic acid. This new acid is optically active and on pyrolysis it yields a volatile acid, presumably hexacosanoic acid. Whether the high molecular weight acid originally contained methoxyl is not known, but it is evident that its hydroxyl group had not been replaced by iodine during the treatment with hydriodic acid. It is probable, in view of the isolation of this new acid, that mycolic acid is a mixture of two acids which are so similar in solubility that they cannot be separated by the methods used in purification.

In order to secure further information concerning the structure of mycolic acid, oxidative degradation was attempted. It was found, however, that a very complex mixture of mono- and dicarboxylic acids was formed when mycolic acid was oxidized with chromic acid. Only three acids could be identified in this mixture, namely stearic acid, *n*-hexadecane-1,16-dicarboxylic acid, and hexacosanoic acid.

The presence of hexacosanoic acid among the oxidation products is of special interest, since this acid is also obtained on pyrolysis. It would seem unlikely that the same group could be the source of hexacosanoic acid by pyrolysis and by oxidation, unless mycolic acid were a coordination compound. It has been shown, however, that mycolic acid is not a coordination compound, because normycolic acid yields no hexacosanoic acid on pyrolysis. This fact would justify the assumption that mycolic acid contains two straight chains, each containing 26 carbon atoms and one of these chains carrying a carboxyl group. The presence of stearic acid among the oxidation products indicates that mycolic acid also contains a straight chain C_{18} group. The dicarboxylic acids probably resulted from secondary oxidations.

EXPERIMENTAL

I. Demethylation of Mycolic Acid. Preparation of Monoiodohydroxynormycolic Acid

The mycolic acid used in these experiments had been prepared as described in a former paper (7). The acid melted at 53–55.5°

and the molecular weight determined by titration was 1316. The methoxyl determination was carried out in a macro-Zeisel apparatus, heated to 150°, and containing from 0.6 to 0.7 gm. of mycolic acid, together with 3 gm. of phenol, 8 cc. of acetic anhydride, 8 cc. of hydriodic acid, sp. gr. 1.70, and 1 cc. of hydriodic acid, sp. gr. 1.96. The volatile iodide was determined quantitatively.

Found, OCH_3 1.33, 1.29

The values for methoxyl agree with those reported earlier (7), namely 1.4 and 1.3. A total of 2.88 gm. of mycolic acid was demethylated and the non-volatile reaction product was collected, washed with water, and dried *in vacuo*. The several lots were combined and dissolved in ether. The ethereal solution was washed first with a dilute solution of sulfur dioxide, and then with water, after which it was dried over sodium sulfate, filtered, and concentrated to a volume of about 75 cc. The iodo compound was precipitated as a white amorphous powder by adding an equal volume of methyl alcohol and cooling in ice water. The precipitate was collected and reprecipitated in the same manner five times. The final product was a snow-white powder which weighed 2.6 gm. It melted at 50.5–52.5°, and remelted at 46–48°, molecular weight by titration 1570, and it contained 10.31 per cent of iodine. The hydroxyl value determined on the methyl ester by Stodola's method (8) was 1.05 per cent. Before the hydroxyl value was determined, the acid was refluxed with benzene and dilute alcoholic potassium hydroxide in order to saponify any acetyl derivative that might have been formed. The free acid was recovered and esterified with diazomethane.

The calculated value for one OH group in a compound having the molecular weight of 1570 is 1.08 per cent and the iodine percentage in a monoiodo compound of the above molecular weight is 8.08 per cent. The equivalent weight found is much too high for a monoiodo acid, but we can give no explanation for this high value.

Hydroxynormycolic Acid—The monoiodohydroxynormycolic acid, 2.36 gm., was dissolved in hot amyl alcohol to which zinc dust and acetic acid were added and the mixture was refluxed for 4 hours, after which the excess zinc was filtered off and washed with amyl alcohol. The filtrate and washings were concentrated

in vacuo nearly to dryness, mixed with water, and extracted with chloroform. The chloroform solution after it had been washed free of acetic acid with water was concentrated to dryness. The residue was dissolved in benzene and after addition of methyl alcoholic potassium hydroxide the solution was refluxed for 6 hours in order to saponify any ester that might have been formed during the reduction. The reaction mixture was concentrated to a small volume by distillation, diluted with ether, and washed first with dilute hydrochloric acid and later with water until the washings were neutral to litmus. The solution was concentrated, mixed with methyl alcohol, and cooled in ice water. The white amorphous precipitate which separated was filtered off, washed with methyl alcohol, and dried *in vacuo*. The substance was free from iodine.

The white powder which weighed 1.8 gm. was dissolved in 120 cc. of ether and the solution was neutralized to phenolphthalein with 0.018 N methyl alcoholic potassium hydroxide, whereupon a small amount of potassium salt separated. After the mixture had stood overnight, the precipitate was filtered off and washed with ether. This substance was examined as will be described later.

The filtrate and washings which contained the ether-soluble potassium salt were combined and washed with dilute hydrochloric acid and with water, after which the solution was concentrated to a volume of about 20 cc. The addition of 50 cc. of methyl alcohol caused a white amorphous precipitate which was collected, washed, and dried *in vacuo*. The snow-white powder weighed 1.49 gm. This substance, which will be called hydroxynormycolic acid, melted at 56–58° and the molecular weight determined by titration was 1284.

The analytical values found are in approximate agreement with the calculated composition of hydroxynormycolic acid.

Analysis—$C_{87}H_{171}O_3$ (1266)
Calculated, C 82.46, H 13.74; found, C 82.30, 82.20, H 13.18, 13.20

The above formula is derived from that of mycolic acid, but like the latter is only tentative.

Hydroxynormycolic acid, which still contains one hydroxyl group, as shown by the presence of one hydroxyl in the iodo compound, when heated under reduced pressure at a temperature of

250–300° was split in the same manner as mycolic acid with the liberation of hexacosanoic acid.

Isolation of a New Acid from the Ether-Insoluble Potassium Salt—The ether-insoluble potassium salt obtained as mentioned above was suspended in ether and shaken with dilute hydrochloric acid until everything dissolved. The free acid was isolated in the usual manner and precipitated from ether solution by the addition of methyl alcohol and cooling. The product was a white amorphous powder and it weighed 0.22 gm. The acid melted at 74–76° without preliminary sintering; molecular weight by titration 1518; $[\alpha]_D^{25}$ in $CHCl_3$ = +4.03°.

Analysis—Found, C 82.69, 82.67; H 13.60, 13.72

The methyl ester, prepared by the action of diazomethane, melted at 63–65°.

Analysis—Found, OH 1.03, mol. wt. (Rast) 1475 ± 57

When the acid was heated under reduced pressure at a temperature of 250–300°, a crystalline acid, presumably hexacosanoic acid, distilled off, but owing to the small amount of material the acid could not be identified.

The exact formula of the new acid cannot be established from the available data, nor is there any certainty as to the purity of the substance. The solubility of its potassium salt in ether is, however, distinctly lower than that of hydroxynormycolic acid. The analytical values found agree approximately with the calculated composition of a hydroxy acid of the formula $C_{104}H_{208}O_3$; calculated, C 82.97, H 13.83, molecular weight 1504.

Preparation of Normycolic Acid

In an attempt to replace both the methoxyl and the hydroxyl groups with iodine, mycolic acid was heated with a more concentrated solution of hydriodic acid. In this experiment 3.75 gm. of mycolic acid were refluxed for 6 hours with 15 gm. of phenol, 25 cc. of hydriodic acid, sp. gr. 1.96, 15 cc. of hydriodic acid, sp. gr. 1.70, and 7 cc. of acetic anhydride. The reaction product was isolated and purified by precipitation from ethereal solution with methyl alcohol. A snow-white amorphous powder was obtained that weighed 4.09 gm. and melted at 41–43°.

Analysis—Found, I 15.4, mol. wt. by titration 1747

The equivalent weight of the substance is much too high for a diiodonormycolic acid. The molecular weight of a diiodonormycolic acid derived from an acid of molecular weight of 1316 should be 1538 and the iodine content should be 16.4 per cent.

The iodo compound, 4 gm., was dissolved in hot amyl alcohol and reduced with zinc dust and acetic acid, after which the reduction product was saponified as described in the first experiment. The recovered acid which was free of iodine was dissolved in ether and the solution was neutralized with methyl alcoholic potassium hydroxide. The ether-insoluble and the ether-soluble potassium salts were separated by filtration and the free acids were liberated in the usual manner.

The acid obtained from the ether-insoluble potassium salt weighed 0.4 gm., melted at 74–76°, and corresponded in properties and composition with the acid of molecular weight 1518 obtained in the first experiment.

The free acid isolated from the ether-soluble potassium salt, after it had been precipitated from ethereal solution by methyl alcohol and cooling, was obtained as a white amorphous powder that weighed 2.7 gm. and melted at 52–54°.

Analysis—

Found, C 83.31, 83.39; H 13.68, 13.62; mol. wt. by titration 1280

In chloroform solution the acid showed no optical activity. The methyl ester, prepared by the action of diazomethane, was tested for hydroxyl by the method of Stodola (8) with completely negative results. The analytical values reported above are in approximate agreement with the formula $C_{87}H_{174}O_2$ (1250); calculated, C 83.52, H 13.92. However, since there are no criteria of purity of the acid, the formula must be considered as tentative.

Pyrolysis of Normycolic Acid

The acid, 1.5 gm., was heated under reduced pressure as described for mycolic acid to a temperature of 250–300° but no volatile acid distilled off. The temperature was raised to about 400° but only a trace of wax-like material condensed in the outlet tube. The contents of the flask were dissolved in ether and the solution was titrated with alcoholic potassium hydroxide with phenolphthalein as indicator, but only 0.49 mg. of potassium hydroxide

was required. It is evident, therefore, that the acid had been almost completely decarboxylated on heating.

The ethereal solution was washed with dilute hydrochloric acid and with water, dried over sodium sulfate, filtered, evaporated to a small volume, and mixed with methyl alcohol. After the solution had been cooled, the precipitate was filtered, washed with methyl alcohol, and dried. The substance melted at 49° after sintering at 36°.

Analysis—C 85.36, H 13.90; mol. wt. (Rast) 1170, 1203; I No. 19.2

II. Oxidation of Mycolic Acid with Potassium Permanganate

An attempt was made at first to oxidize mycolic acid in boiling acetone solution with potassium permanganate, but this procedure did not yield any oxidation products that appeared at all promising or helpful for the elucidation of structure. The principal product that was recovered differed but slightly in melting point and equivalent weight from the original acid. A small quantity of acids which had an equivalent weight of 741 and melted at 61–68° was isolated but the material was non-crystalline and was apparently a mixture. It is evident that oxidation with permanganate was not sufficiently drastic to break down the large mycolic acid molecule into smaller recognizable units. This method of oxidation was therefore discontinued.

Oxidation of Mycolic Acid with Chromic Acid

It was found in preliminary experiments that when mycolic acid was oxidized in glacial acetic acid with chromic acid it was completely broken down, with the formation of a complex mixture of lower fatty acids.

The following procedure was used in the final oxidation experiments. The mycolic acid, 3.0 gm., in 600 cc. of boiling glacial acetic acid, contained in a flask provided with a ground joint condenser, was dispersed into finely divided globules by shaking and the emulsified condition was maintained by vigorous boiling. To the mixture was added gradually during a period of 45 minutes a concentrated solution of 7.8 gm. of chromic acid dissolved in hot glacial acetic acid, after which the reaction mixture was diluted with water and distilled with steam. However, mere traces of steam-volatile material were obtained, and were not further examined.

The non-volatile oxidation products were extracted with ether and the extract, after it had been washed free of acetic acid with water, was separated into neutral and acid components. A total of 24.7 gm. of mycolic acid was oxidized and yielded 21.1 gm. of fatty acids.

The acids were esterified in ether solution with diazomethane and the methyl esters were subjected to fractional distillation in a high vacuum. Two fractionations were made in a flask provided with a column of the Widmer type. The first fractions were colorless oils at room temperature while the later fractions were semisolid and partly crystalline. A gradual rise in the boiling point was observed without any sharp change in properties. Three additional fractionations through a column similar to that recommended by Klenk (9) did not yield any pure esters. After several further fractionations through a column similar to that described by Craig (10) a series of 60 fractions was collected, several of which had the same boiling and melting points. However, none of these fractions was homogeneous. Further purification was effected by crystallization from acetone until the melting points remained constant, but very small amounts of the pure esters were obtained. By far the greater quantity of the esters remained unidentified and represented a very complex mixture that could not be separated into homogeneous compounds either by distillation or by crystallization. The three ester fractions described below were the only pure substances that could be isolated.

Methyl Stearate—Three ester fractions from the sixth fractionation which melted at 28–28.5° were combined and crystallized from acetone until the melting point was constant at 38°. The purified ester, 0.25 gm., gave on saponification the equivalent weight of 297. Methyl stearate requires an equivalent weight of 298.

The free acid was isolated and crystallized from acetone and was obtained as colorless, thin plates. The acid melted at 68.5° and there was no depression when mixed with an authentic sample of stearic acid.

Analysis—$C_{18}H_{36}O_2$ (284).	Calculated.	C 76.05, H 12.67
	Found.	" 76.10, " 12.85

Methyl n-Hexadecane-1,16-Dicarboxylate—Three fractions from the fifth fractionation which melted at 38–39° were combined,

total weight 0.45 gm., and recrystallized from aqueous acetone until the melting point was constant at 55–56°. The yield was 22 mg. as colorless needles. On saponification the ester had an equivalent weight of 172. The free acid on crystallization from aqueous acetone gave fine colorless needles which melted at 121–122°.

Analysis—$C_{18}H_{34}O_4$ (314).	Calculated.	C 68.79, H 10.82
	Found.	" 68.81, " 10.96

The melting points of the ester as well as that of the free acid correspond with those reported for *n*-hexadecane-1,16-dicarboxylic acid (11).

Methyl Hexacosanoate—Four fractions from the fifth fractionation which melted between 53–55° were combined, total weight 0.6 gm., and recrystallized from acetone until the melting point was constant at 62–62.5°. The purified ester, 0.35 gm., gave on saponification an equivalent weight of 409. The equivalent weight of methyl hexacosanoate is 410. The free acid on crystallization from acetone separated as colorless thin plates and melted at 87–88°. There was no depression of the melting point when the acid was mixed with pure hexacosanoic acid, m.p. 88–89°.

Analysis—$C_{26}H_{52}O_2$ (396).	Calculated.	C 78.78, H 13.12
	Found.	" 78.74, " 13.15
		" 78.69, " 13.11

SUMMARY

1. Mycolic acid yields on demethylation with hydriodic acid a monoiodo compound which on reduction gives hydroxynormycolic acid. The latter on pyrolysis is split in the same manner as mycolic acid into normal hexacosanoic acid and a non-volatile residue.

2. Diiodonormycolic acid, formed when mycolic acid is refluxed with concentrated hydriodic acid, yields on reduction normycolic acid. The latter acid is decarboxylated on pyrolysis and no volatile acid is obtained.

3. A small amount of a hydroxy acid of the approximate formula $C_{104}H_{208}O_3$ was separated from hydroxynormycolic acid and from normycolic acid, thus indicating that mycolic acid is a mixture of two acids.

4. When mycolic acid is oxidized with chromic acid, a complex mixture of mono- and dicarboxylic acids is produced. The only

acids that could be identified among the oxidation products were stearic acid, *n*-hexadecane-1,16-dicarboxylic acid, and *n*-hexacosanoic acid.

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COMPARATIVE METABOLISM OF GLUCOSE AND FRUCTOSE IN THE RAT

By N. R. BLATHERWICK, PHOEBE J. BRADSHAW, MARY E. EWING, AND SUSAN D. SAWYER

(*From the Biochemical Laboratory of the Metropolitan Life Insurance Company, New York*)

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Much is known concerning the metabolism of glucose and fructose but one has only to read the review by Deuel (1) to recall that many problems, especially regarding fructose, await solution or clarification. Our interest in this field was quickened when it was found that persons having essential fructosuria do not respond with the usual increase in blood lactic acid observed in the normal individual when either fructose or sucrose is given. One hypothesis concerning the defect in metabolism giving rise to fructosuria is that the liver is unable to effect a stage or stages in the metabolism of fructose which the normal person is able to consummate. It seemed possible that the failure to find an increase in the content of the blood lactic acid after the administration of fructose to persons with essential fructosuria might be related to the suggested liver defect. This view obtains support from the experiments described below, which indicate that the liver of the normal animal is intimately involved in the production of lactic acid during the metabolism of fructose.

White rats of both sexes varying in age from 93 to 111 days and weighing from 175 to 350 gm. were fasted for 24 hours and then given 1.5 cc. of either 50 per cent glucose or fructose solution by stomach tube. After an absorption period of 1 hour, the rats were anesthetized with sodium amytal and then their skinned hind legs and livers were frozen *in situ* with CO_2 snow. The procedure of obtaining the tissues and our methods for determining the tissue carbohydrates have been described (2). Lactic acid, glycogen, and total reducing substances of liver and muscle were determined.

The mean values were analyzed for statistical significance by the *t* test of Fisher (3) for small series of observations.

The values given in Table I are so arranged that a direct comparison of the effects of the administration of fructose can be made with those resulting from the giving of glucose. It is seen that 1 hour after the sugars are given the following tissue constituents of the rats fed fructose were significantly increased: liver lactic acid, 37.1 and 22.2 mg. per cent for fructose and glucose, respectively; muscle lactic acid, 34.7 and 22.9 mg. per cent; and muscle glycogen, 658 and 573 mg. per cent. The values for liver glycogen and for total reducing substances of muscle and liver of the animals fed fructose were also greater but not enough to be significant.

TABLE I

Carbohydrate Content of Tissues of Rats Given Glucose and Fructose

The results are expressed in mg. per 100 gm. The values for glycogen are given in terms of glucose. The figures in bold-faced type represent values for the rats given fructose.

Substance determined	Maximal	Minimal	Mean	Standard deviation of mean	No. of rats	
					Males	Females
Glycogen, liver	1798	770	1219	124	4	4
" "	**2000**	**795**	**1528**	**153**	**3**	**5**
" muscle	682	501	573	27	4	4
" "	**735**	**613**	**658***	**19**	**3**	**5**
Lactic acid, liver	30.4	14.6	22.2	2.2	4	4
" " "	**66.5**	**19.0**	**37.1***	**6.2**	**3**	**5**
" " muscle	30.5	16.1	22.9	1.8	4	4
" " "	**43.1**	**16.6**	**34.7***	**3.0**	**3**	**5**
Total reducing substances, liver	217	145	175	9.5	4	4
" "	**298**	**155**	**204**	**22.3**	**2**	**5**
Total reducing substances, muscle	37.4	22.0	29.1	2.1	4	4
" "	**34.3**	**20.4**	**27.0**	**1.7**	**2**	**5**

* Statistically significant by the *t* test of Fisher.

DISCUSSION

The results show that 1 hour after the sugars were given the lactic acid content of the liver and the muscles of the rats fed

fructose was 67 and 52 per cent greater than in the case of the animals to which glucose was administered. At the same time, the liver glycogen of the former group was increased by 15 per cent. The data show definitely that these tissues were actively engaged in the metabolism of fructose, with the production of lactic acid. It is well known that the blood lactic acid increases after the ingestion of fructose and is thus carried to the various tissues. The place of origin of the lactic acid in these experiments cannot be given but from the studies of Wierzuchowski and Sekuracki (4) it seems plausible to assign the major rôle to the liver. These workers found that the administration of fructose to the dog resulted in a marked formation of lactic acid in the liver, in contrast to the utilization or conversion of this substance when galactose, glucose, and maltose were given. Their results were based upon analysis of the blood entering and leaving the liver, while ours rested upon direct determinations made on the organ.

Our findings are pertinent to the discussion regarding the greater rise in the respiratory quotient following the ingestion of fructose as compared with some other sugars. It is held by many that this effect is caused by an upset in the acid-base balance due to the production of lactic acid which results in a blowing off of CO_2 from the blood and other tissues. Others believe that the higher respiratory quotient is not due to the production of lactic acid but that it represents a more rapid oxidation of fructose. The observations herein recorded are considered to give definite weight to the view that lactic acid production is largely responsible for the elevation of the respiratory quotient.

SUMMARY

The administration of fructose by stomach tube to rats resulted in more than a 50 per cent increase in the lactic acid content of the liver and the muscles as compared with the results when glucose was given. This intermediary production of lactic acid from fructose is considered to be added evidence for the view that lactic acid is largely responsible for the higher respiratory quotients observed after the ingestion of this sugar.

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A MODIFIED SILVER COBALTINITRITE METHOD FOR POTASSIUM DETERMINATION*

By JOHN E. HARRIS

(*From the Department of Obstetrics and Gynecology and the Department of Biochemistry, State University of Iowa, Iowa City*)

(Received for publication, July 30, 1940)

The determination of potassium as the silver cobaltinitrite salt was first adapted to biological materials by Breh and Gaebler (1), and Truszkowski and Zwemer (2) have offered modifications of the original method. In all these procedures the potassium ion is precipitated from chloride-free, Folin-Wu filtrates, but Cumings (3) has recently employed serum ash solution. Robinson and Putnam (4) have adapted the precipitation to the determination of potassium in water and Ismail and Harwood (5) to soil analyses.

Burgess and Kamm (6) state that the silver cobaltinitrite reagent will detect 0.5 part of potassium per million at 15°; the sensitivity decreases as the temperature is raised, being about 1 part per million at 20° and 5 parts per million at 30°. This property makes possible the use of protein-free filtrates in the lower temperature range. In our experience, however, the existing methods (1, 2) were inadequate, their accuracy being no greater than ±3 to ±5 per cent, while frequently greater deviations were noted. Serious sources of error appeared to be an inconstant composition of the precipitate, loss of precipitate during washing, and the presence of excess silver in the chloride- and protein-free filtrates, giving rise to high values. Modifications designed to remedy these and other errors have been devised. The new procedure is less time-consuming and appears to be accurate within ±2 per cent.

Reagents—

1. Potassium standard. The solution employed contains 0.1

* From a dissertation submitted to the Faculty of the Graduate College of the State University of Iowa in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

mg. of potassium per ml. (0.2228 gm. of potassium sulfate in 1 liter of solution). 1 ml. of each of the protein precipitants is added to 10 ml. of the standard and diluted to 50 ml.[1] 4 ml. of the diluted solution, containing 0.08 mg. of potassium are used as the standard.

2. Precipitating reagent. The reagent of Breh and Gaebler is used except that the sodium cobaltinitrite and silver nitrate are mixed at the time of preparation rather than immediately before use. 210 ml. of a solution of 120 gm. of sodium nitrite in 180 ml. of water are added to a solution of 25 gm. of cobalt nitrate in 50 ml. of water and 12.5 ml. of glacial acetic acid. The mixture is aerated for 1½ to 2 hours. 0.1 volume (usually 28 ml.) of 40 per cent silver nitrate is added and the precipitate is dissolved by vigorous shaking. The reagent is stored in the refrigerator, but, prior to use, it is warmed to room temperature and filtered. It should be reaerated weekly and discarded after 1 month.

3. Protein precipitants, $\frac{2}{3}$ N sulfuric acid and 10 per cent sodium tungstate.

4. 1.80 per cent silver nitrate.

5. Wash reagent, 2 parts of alcohol, 1 part of ether (peroxide-free), and 2 parts of water. The solution should be protected from evaporation.

6. 0.2 N sodium hydroxide.

7. 10 per cent acetic acid.

8. Diazotization materials, 1 volume of 0.5 per cent α-naphthylamine in 30 per cent acetic acid and 2 volumes of 0.5 per cent sulfanilic acid in 30 per cent acetic acid (freshly mixed). The sulfanilic acid solution appears stable, but the α-naphthylamine becomes colored in a week or two and must be discarded. The solution will keep longer if stored in the refrigerator. Robinson and Putnam suggest mixing the materials at the time of preparation, but the method recommended is more economical.

Procedure

1 ml. of serum or plasma is added to 7 ml. of water in a centrifuge tube, followed by 0.5 ml. of $\frac{2}{3}$ N sulfuric acid, 0.5 ml. of 10

[1] When the salt is precipitated from standard or ashed solutions, the solid tends to cling to the side of the tube. The presence of a small amount of tungstic acid avoids this difficulty, possibly because the precipitation is slower. For this reason the protein precipitants are added to the standard and to ashed solutions when these are employed.

per cent sodium tungstate, and 1 ml. of 1.80 per cent silver nitrate, with thorough mixing after each addition. The tube is allowed to stand for 15 minutes and centrifuged.

4 ml. of the filtrate are placed in a 15 ml., graduated centrifuge tube and 1 ml. of 95 per cent alcohol is added, with thorough mixing. 4 ml. of the diluted standard are treated in the same manner. There is insufficient filtrate for two 4 ml. samples. If it is desired to carry out duplicate determinations on 1 ml. of serum, 3 ml. aliquots of the filtrate and diluted standard are taken. 1 ml. of water and 1 ml. of alcohol are added and the treatment continued as with the 4 ml. samples.

For whole blood analysis, 0.2 ml. of the sample is added to 8.3 ml. of water, followed by 0.5 ml. of $\frac{2}{3}$ N sulfuric acid, 0.5 ml. of 10 per cent sodium tungstate, and 0.5 ml. of 1.80 per cent silver nitrate. 2 ml. of the filtrate are taken for analysis, an equal volume of water and 1 ml. of alcohol being added and treatment continued as with the plasma samples. The method is applicable to urine analyses after ashing.

The tubes are placed in a water bath maintained at 20°. After 5 minutes 2.0 ml. of the precipitating reagent are added and the contents mixed by striking the bottom of the tubes, which are replaced in the bath and allowed to stand for $\frac{1}{2}$ hour. At temperatures much higher than 25° the nitrite to potassium ratio in the precipitate is not constant; if the temperature is too low (16°) or the silver nitrate concentration too high, long, needle-like silver nitrite crystals may precipitate. In this case the precipitation must be repeated.

Upon removal from the bath the tubes are centrifuged for 10 minutes at about 2800 R.P.M. The supernatant fluid is removed to 0.2 ml. with suction, by means of a capillary glass tube. 7 ml. of the wash reagent are added, being blown down on the precipitate in such a way as to mix it completely with the wash solution.[2] After 10 minutes centrifuging the supernatant fluid is decanted, and, without being inverted again, the tubes are allowed to drain

[2] The proper choice of wash reagent is important. In spite of the fact that Burgess and Kamm (6) state that the precipitate "exhibits a marked tendency to pass into the colloidal condition when washed with water," certain investigators (1, 2) use water for this purpose. Water likewise has the disadvantage of causing flotation of the precipitate, even when octyl or caprylic alcohol is used. These disadvantages have been overcome by using a mixture of water, alcohol, and ether.

on an absorbent material for 5 minutes. The lips of the tubes are wiped and the washing repeated. It is convenient, when a large number of determinations are being carried out, to have two sets of tubes, one centrifuging while the other is being drained and prepared. Both may be compared with one standard.

After the precipitate has been washed and drained for the second time, the lips of the tubes are again wiped. 10 ml. of 0.2 N sodium hydroxide are added to each by blowing directly upon the precipitate, mixing it completely with the solution. Occasionally some of the material clumps in the bottom of the tube, but this has not been observed to vitiate the results. The tubes are placed in a bath of boiling water for 10 minutes, removed, and allowed to cool. The cooling may be hastened by placing the tubes under the tap. The volume is then made up to 10 ml. with distilled water, the contents mixed, and the tubes centrifuged for 5 minutes. The use of graduated centrifuge tubes has been found satisfactory. As an alternative procedure, the cooled, alkaline mixture may be quantitatively transferred to a 25 ml. volumetric flask, made up to volume with water, and filtered.

2 ml. of the centrifugate (or 5 ml. of the filtrate, if the volume is made to 25 ml.) of both the standard and the unknown solutions are added to 15 ml. of 10 per cent acetic acid in 50 ml. volumetric flasks. 3 ml. of the diazotization materials are added, the contents mixed, and the color allowed to develop for 5 minutes. The solutions are made up to volume with 10 per cent acetic acid, mixed by inversion, and the readings made, with the standard set at 15 mm. If the reading of the unknown is above 19 or below 10, the color must be redeveloped, with more or less of the centrifugate as the situation demands.[3] Readings should be made within ½ hour after dilution. A blue light filter is used in our laboratory.

[3] A series of solutions containing various concentrations of sodium nitrite was prepared and the color developed as described. The reading was found to be directly proportional to the concentration of the nitrite only within the limits specified. It is important to let the color develop a full 5 minutes before dilution. Color development in the dilute solution proceeds more slowly and is not complete in 10 minutes. The precaution advised by Truszkowski and Zwemer of changing the standard after six readings does not appear to be necessary.

Calculations—$S/U \times 40/A$ = mg. of potassium in 100 ml. of plasma or serum, when S = reading of standard, U = reading of unknown, and A = ml. of centrifugate of unknown solution taken for color development. (When the final volume before color development is 25 ml., the value is multiplied by 2.5.)

Comment

One of the chief objections to the determination of potassium as sodium potassium cobaltinitrite is that the composition of the salt varies with the conditions of precipitation (7). This holds

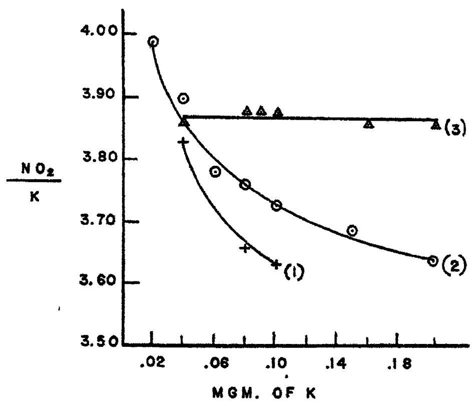

FIG. 1. Variation of the nitrite to potassium ratio with the potassium concentration at 0° and 20°. Curve 1, precipitation at 0° without alcohol; Curve 2, precipitation at 0° with alcohol; Curve 3, precipitation at 20°. All points are the averages of two or more determinations.

true also if the silver cobaltinitrite reagent is used, varying amounts of $AgK_2Co(NO_2)_6$ or $Ag_2KCo(NO_2)_6$ (and possibly some of the sodium salts) being obtained, depending especially upon the proportions of silver and potassium and the temperature.

Truszkowski and Zwemer, unable to obtain satisfactory results with precipitation at room temperature during the summer heat, precipitated the salt overnight in an ice bath in the refrigerator; to prevent the precipitation of silver nitrite the silver concentration of the precipitating reagent was reduced. Employing this technique, we have determined the nitrite concentration of the

precipitate from known amounts of potassium. The results are given in Fig. 1, Curve 1; alcohol was added before precipitation as in the present method in another series of determinations shown in Curve 2. In both curves the nitrite to potassium ratio[4] increases as the potassium concentration decreases, the silver remaining constant. Reducing the silver concentration while that of potassium is kept constant results in a lower ratio, in harmony with the above facts. This is in accordance with the observations of Robinson and Putnam. However, Ismail and Harwood found no such discrepancy.

The nitrite to potassium ratio in the precipitate formed at 20° in the present procedure is constant over a wide range of potassium concentrations (Fig. 1, Curve 3). This constancy, as confirmed in similar tests, is maintained fairly well at 15° and 25°. Nevertheless, although the difference is less than 2 per cent, the ratio at 15° is slightly greater for low (0.04 mg.) than for higher (0.08 mg.) potassium content, while at 25° the reverse is true. Moreover, the nitrite to potassium ratio is higher at lower temperatures, being 4.06 at 15°, 3.87 at 20°, and 3.75 at 25° (with 0.08 mg. of potassium), all other conditions remaining constant.

At 30° or above the ratio is significantly increased at the higher potassium concentrations. The ratio at 30° was found to be 3.74 when 0.08 mg. of potassium was taken and 3.63 with 0.04 mg., while at 37° the nitrite to potassium ratios were 3.39 and 2.74, respectively. This is in contradistinction to the results at 0°, at which the higher ratio was obtained with the lower potassium concentration. Incomplete precipitation at the higher temperatures is suggested. The pivotal temperature appears to be 20°.

Fairly accurate control of the amount of silver nitrate added in the preparation of the protein- and chloride-free filtrate appears necessary. However, a slight excess of either chloride or silver has no effect. Thus chloride values ranging from 450 to 850 mg. per 100 ml. (as NaCl) appear to be well tolerated when the amount of silver nitrate specified (18 mg. to 1 ml. of plasma or serum) is added. Beyond these limits it is well to increase or decrease the

[4] The mole ratio of the nitrite concentration as determined by comparison with standard sodium nitrite solution to potassium concentration taken for analysis.

amount of silver nitrate added to the plasma as the situation demands. High values result if the silver nitrate concentration of the filtrate is much greater than 0.05 per cent, the value theoretically obtained with the lower chloride limit mentioned above.

The presence of the ammonium ion in the sample gives rise to high values. For example, a solution containing 20 mg. per cent of potassium and 1.8 mg. per cent of ammonia nitrogen (as ammonium acetate) showed a measured potassium value of 22 mg. per cent. Thus the results of Scudder, Corcoran, and Drew (8) pertaining to the effect of ammonia on the diffusion of potassium from erythrocytes should be accepted with reservation, since they failed to remove added ammonia from plasma samples before the determination of potassium by a silver cobaltinitrite method. Favorable comparison of values on ashed and filtrate specimens indicates that the small amount of ammonia present in freshly drawn serum or plasma is insufficient to interfere.

TABLE I

Recovery of Potassium from Known Solutions

Potassium taken	No. of determinations	Recovery, average value	Maximum deviation
mg. per cent		*mg. per cent*	*mg. per cent*
50.0	4	49.5	49.2 -49.6
40.0	3	40.0	39.5 -40.5
25.0	4	24.6	24.4 -25.0
22.5	2	22.5	
20.0	7	20.1	19.9 -20.3
15.0	2	15.0	
10.0	9	10.0	9.85-10.13

Results

A series of determinations with known concentrations of potassium were carried out according to the present procedure. The results are given in Table I. It will be observed that the greatest error is 1.6 per cent. Thus a conservative estimate of the method's accuracy is ± 2 per cent.

It seemed desirable to carry out parallel determinations on plasma samples according to both the present procedure and a

chloroplatinate technique. For the latter, the method of Consolazio and Talbott (9) was used after slight modification and verification with known potassium solutions. Determinations were made in duplicate. Table II indicates that the present method gives values slightly higher than those obtained with the chloroplatinate method. The variations are within the limits of

TABLE II

Comparison of Parallel Determinations of Serum Potassium by Chloroplatinate Method and Method Described

The values are given in mg. per 100 cc. of serum.

Determination No.	Chloroplatinate method	Method described
1a	18.3	18.0
1b	19.1	18.3
1c	17.1	17.1
2	16.1	16.3*
3†	8.10	7.84*
4a	17.0	17.2
4b	16.7	16.8
4c	18.7	19.2
5a	22.9	23.4
5b	23.3	24.1
5c	25.5	25.6
6a	15.5	15.4
6b	16.7	17.1
6c	17.1	17.6
Average	18.0	18.1

The three values for the same individual represent samples taken before, during, and after transfusion with preserved blood.

* Not in duplicate.

† Diluted plasma of fresh blood.

error of the methods but are consistent. The discrepancy can perhaps be ascribed to differences in treatment before precipitation; the chloroplatinate technique involves ashing, while in the present procedure protein-free filtrates are used. The fact that the values of ashed specimens as determined by the described method are generally 1 to 2 per cent lower than the corresponding filtrate values is in harmony with this suggestion.

SUMMARY

A procedure for the determination of potassium as potassium silver cobaltinitrite has been described. In the temperature range studied, it appears that 20° is the most favorable for precipitation, since at that point the nitrite to potassium ratio remains constant over a wide range of potassium concentrations. Water as a wash reagent has been replaced by a solution of water, alcohol, and ether. The method appears to be accurate within ±2 per cent.

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CHEMISTRY OF THE CHICK EMBRYO

III. DISTRIBUTION OF DIPEPTIDASE IN THE CEPHALIC REGION OF THE THREE DAY EMBRYO*

By ALBERT H. PALMER and MILTON LEVY

(*From the Department of Chemistry, New York University College of Medicine, New York*)

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During the early stages of incubation the cephalic region of the chick embryo increases in weight much more rapidly than does the body. It has been suggested (3) that the weight accumulation coefficient during the first phase of weight accumulation is largely related to the increase in cephalic tissue, increase in body tissue playing a minor rôle. Quantitative information regarding the distribution of enzymes in this region should be of interest. The present paper deals with the distribution of alanylglycine dipeptidase.

Materials and Methods

The source of eggs, incubation practice, and estimation of dipeptidase have been described (3, 5).

Because we wished to estimate an enzyme in microtome sections, ordinary methods of fixing, staining, and imbedding could not be used; neither did we find it possible to remove frozen sections from the knife for tissue measurements. We observed, however, that while the embryo was being cut on the microtome, the boundaries between the various parts of the embryo were clearly visible on the freshly cut surface of the ice block. We developed the following technique for obtaining a record of the distribution of each kind of tissue in the sections.

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A microscope tube was mounted on the knife carriage of a clinical freezing microtome. The mounting was adjustable in the horizontal plane, so that the microscope could be set over any part of the freezing stage and the regular rack and pinion adjustments of the microscope were retained. A Zeiss Miflex camera was mounted on the microscope with a roll film carrier, enabling us to take sixteen pictures on a roll of No. 127 Panatomic film. The entire microscope and camera moved with the knife and were set so that the microscope was above the specimen on the extreme back position of the knife. Illumination was provided by a 200 watt lamp through a condenser and $CuSO_4$ filter solution. The light impinged at an angle of about 30° on the cut surface. With the 32 mm. objective ordinarily used, we found 0.5 second exposure to be adequate. After the specimen had been frozen in position and the surface cut to some initial point, the microscope was brought to position and focused. (A magnifying focusing device is much more satisfactory than the usual ground glass plate.) The microtome was operated until the initial point desired was reached and an exposure was made immediately after cutting the surface; delay allowed the accumulation of frost which obscured the picture. The next forward movement of the knife cut the desired section.

Preparation and Mounting of Specimen—Embryos were removed from the egg as previously described (3). All embryonic membranes except a small strip caudally were cut away.

A cylinder of tin-foil was prepared by rolling a narrow strip on an 8 mm. vaselined glass rod and fastened together with a bit of Scotch tape. The cylinder was set upright on the microtome stage and a few drops of 0.05 per cent methylene blue in 0.9 per cent NaCl placed in it. This was frozen and served to hold the cylinder in place during subsequent operations. The use of methylene blue in the mounting fluid sharpened the outline of the embryo and reduced subsurface reflections in the ice. It did not interfere with subsequent dipeptidase estimations.

The embryo was washed with the same methylene blue saline, lifted by the bit of membrane remaining, and, if properly prepared, oriented in a vertical position with the head down. It was carefully lowered into the cylinder and held by one operator while the

other added methylene blue saline and operated the CO_2 valve so that the embryo was frozen within the tin-foil cylinder imbedded in ice. The tin-foil was stripped off. Unwanted sections of the embryo were cut and discarded. If transverse sections were wanted, cutting and photographing were continued without change of position. If sagittal or frontal sections were required, the exposed transverse section was photographed for orientation, a flat side shaved on the ice cylinder, and the cylinder broken free from the stage. It was then refrozen to the stage on its flat surface. After readjustment of the microscope and camera, sectioning was continued. Our general practice was to discard every other section. At the end of each run a photograph of a mm. scale was made without changing the setting. This served as a measure of magnification. The sections were always 0.05 mm. thick.

The section melted almost immediately after cutting. The drop of material representing the section was picked up with a 2.5 mm. circle of filter paper held in forceps designed for removing foreign objects from the external ear canal. The paper circles were cut with a saddler's punch. The paper took up all of the material cut and was then transferred to a tube containing 8.5 c.mm. of 30 per cent glycerol. From this point the estimation of dipeptidase activity followed the usual procedure. From the data the activity of each section was calculated and converted to dipeptidase units. Blanks were obtained by using sections at regular intervals with addition of acid before substrate. Intermediate blanks were estimated by interpolation between these.

Estimation of Amounts of Tissue—Projection prints of the negatives and the scale were made. The outlines of the various tissues were marked on this and their areas measured with a planimeter. Comparison with a measured area on the scale allowed the calculation of the volumes of each kind of tissue, ectoderm (E), mesenchyme (M), and cephalic fluid (C), from the areas and the thickness of the sections (0.05 mm.). These volumes were plotted against section number and a line drawn through the points as in Fig. 1 (lower curves marked E, M, C). The volumes of tissue present in each section used for dipeptidase were read from these diagrams at a point half way through the section. We thus had measurements of the volumes of ectoderm, mesenchyme, and ce-

phalic fluid as well as the amount of dipeptidase in each section. Fig. 1 illustrates the data obtained by different directions of sectioning.

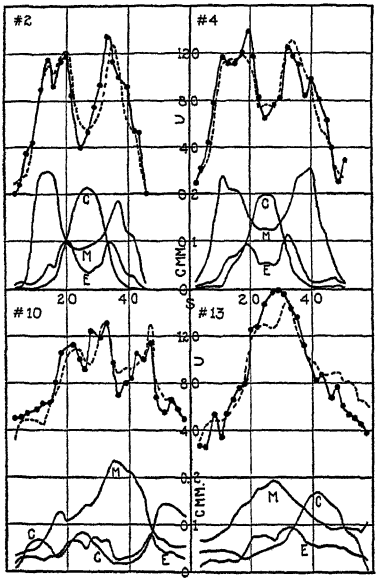

FIG. 1. The figures on the abscissa represent the numbers of the sections; the ordinates represent dipeptidase units per section (upper curves), or c.mm. of tissue per section (lower curves); E ectoderm, M mesenchyme, C cephalic fluid. The numbers in the upper left-hand corners are the numbers of the embryo in Table I. The dots are experimental values. The dash curves are calculated from $U = f_1E + f_2M$, as defined in the text. The direction of sectioning was sagittal (Embryos 2 and 4), frontal (Embryo 10), or transverse (Embryo 13).

DISCUSSION

As a hypothesis for the treatment of the data we assume that each type of tissue has a characteristic enzyme concentration. This assumption leads to the equation, $U = f_1E + f_2M + f_3C$, where U is the total enzyme in units and E, M, and C are the volumes in c.mm. of ectoderm, mesenchyme, and cephalic fluid, respectively, in each section, and f_1, f_2, f_3 are characteristic concentrations of dipeptidase in units per c.mm. Direct study of isolated

cephalic fluid showed f_3 to be 0. It had no enzyme, nor did it inhibit or activate an enzyme extract. The equation reduces to $U = f_1E + f_2M$. Estimation of f_1 and f_2 is more difficult. No sections could be obtained in which only one kind of tissue was present in significant quantities. It becomes necessary to solve the series of simultaneous equations for each embryo for f_1 and f_2. This can be done by use of the method of least squares. However, the result will be vitiated by the presence in the series of

TABLE I

Distribution Constants of Dipeptidase in Chick Embryo Heads

Embryo No.	Direction of sectioning	Linear correlation coefficient E vs. M	f_1	f_2	Average per section	Average error per section
			units per c.mm.	*units per c.mm.*		
1	Sagittal	0.56	954	273	86	±9
2	"	0.39	916	255	72	±8
3	"	0.53	1135	358	75	±10
4	"	0.45	617	341	80	±9
5	"	0.61	597	367	66	±7
6	"	0.68	753	368	73	±12
7	Frontal	0.29	1109	202	72	±10
8	"	0.40	990	225	81	±5
9	"	0.28	1244	153	74	±10
10	"	0.14	1233	225	83	±6
11	Transverse	0.84	353	640	86	±7
12	"	0.78	476	470	68	±9
13	"	0.84	1045	283	86	±9
14	"	0.80	575	420	78	±23
Average, Embryos 1–10.			955	277		

sections of proportional amounts of ectoderm and mesenchyme. This proportionality can be judged by means of the correlation between E and M. If the correlation is perfect, *i.e.* the correlation coefficient is 1, then the data are not suitable for effective characterization of f_1 and f_2. If the correlation is low, a more or less accurate differentiation of f_1 and f_2 can be made. In transverse sections we find the correlation coefficient to be high, 0.78 to 0.84; in frontal sections it is low, 0.14 to 0.40; and in sagittal

sections, intermediate, 0.39 to 0.68. With this in mind, we have excluded from the averages all the transverse sectionings.

Table I shows the values of f_1, f_2, and correlation coefficient between E and M in each embryo head as well as the average U per section and the probable error of the calculated values. In Fig. 1 the calculated values of U obtained from the factors and E and M of each section are shown.

The averaged values of f_1 and f_2 (Table I) show that the concentration of dipeptidase is about 3 times as great in ectoderm as in mesenchyme. While we know that cells in the ectoderm are more closely packed than in the mesenchyme, we cannot at this time form an opinion as to whether f_1 and f_2 are proportional to the cell concentrations in the respective tissues. Changes in the factors during development may serve as a clue to the metabolic tools and processes of the various types of tissue.

With marine ova Holter, Lanz, and Linderstrøm-Lang (1) have shown that the first three cleavages are unaccompanied by change in the total dipeptidase and the resultant eight blastomeres contain one-eighth of the original dipeptidase each. Similarly, Holter and Lindahl (2) have shown that up to the pluteus stage in *Paracentrotus lividus* the enzyme concentration remains the same in the various parts of the embryo. The marine ova which have been studied represent a much earlier stage in development than our chick embryos, assuming comparability of the two kinds of embryos. As Linderstrøm-Lang (4) remarks, differences in distribution must occur, because the dipeptidase is not equally distributed in the grown animal. It may be that as long as growth occurs by division of the initial cell without increase of total substance, each cell will have its proportion of the total dipeptidase originally present. When growth occurs by multiplication, with increase of substance, each new cell formed should have its complement of newly formed enzymes.

SUMMARY

Dipeptidase in the cephalic region of the chick embryo is distributed in concentrations characteristic of each kind of tissue. Ectoderm contains about 3 times the concentration of dipeptidase in mesenchyme and the cephalic fluid contains none of the enzyme.

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THE ACTIVATION OF COCARBOXYLASE BY THIAMINE*

By M. A. LIPTON and C. A. ELVEHJEM

(*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*)

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The discovery of the identity of cocarboxylase and thiamine pyrophosphate (1) has stimulated considerable research upon the distribution and function of this compound in biological materials. Such research requires a convenient and accurate method for the determination of this compound. The most widely used method is that in which cocarboxylase is determined by measuring its activity as the coenzyme for yeast carboxylase. Yeast washed with alkaline phosphate buffer is unable to decarboxylate pyruvic acid, but upon the addition of cocarboxylase and magnesium or manganese, activity is restored. Lohmann and Schuster (1) have described optimum conditions for the test and have demonstrated that the method is highly specific since neither thiamine nor thiamine monophosphate shows activity in this test. The assay procedure of Lohmann and Schuster has been employed by most workers who have studied the distribution of cocarboxylase or the mechanism of its synthesis from thiamine by yeast, animal, or bacterial enzymes (2–5).

Recently, Ochoa and Peters (6) have shown that, although thiamine is inactive as the coenzyme for bakers' yeast carboxylase, it nevertheless augments the activity of small amounts of cocarboxylase. As a result of this finding, they consider previous methods for the quantitative determination of cocarboxylase to be invalid, particularly when free thiamine is present with cocarboxylase. Since thiamine is necessarily added to enzyme systems in which the synthesis of cocarboxylase is being studied,

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Ochoa and Peters feel that the experiments of various workers (2–4) who have neglected to account for the stimulatory effect of thiamine upon cocarboxylase have been incorrectly interpreted.

We have previously shown that cocarboxylase may be synthesized from thiamine by enzymes present in alkaline washed brewers' yeast, provided that hexose diphosphate and boiled tissue extracts are present (2). In this work the amount of cocarboxylase present after synthesis was determined by the method of Lohmann and Schuster (1). The work has been criticized, therefore, on the grounds that the increased decarboxylation of pyruvic acid noted in our experiments was due not to the synthesis of cocarboxylase, but rather to the activation of cocarboxylase present in our boiled tissue extracts by the thiamine which was added.

Reference to our published data (2) shows that the phenomenon noted by the English workers was not observed in our experiments. Thus, when thiamine was added to a system capable of synthesizing cocarboxylase in the presence of an inhibitor of the synthesis (sodium iodoacetate), no increase in the rate of decarboxylation of pyruvic acid occurred. Since iodoacetate has been shown to affect the synthesis but not the activation of cocarboxylase by thiamine (2, 6), it will be apparent that activation did not occur in our work. It was also shown (7) that an incubation period of 30 to 60 minutes was required for appreciable synthesis of cocarboxylase. Experiments in which thiamine was added to a system which could synthesize cocarboxylase showed that if the enzymes required for synthesis were inactivated immediately by boiling the amount of cocarboxylase measured by our method did not increase. These two experiments demonstrated that the activation of cocarboxylase by thiamine described by Ochoa and Peters (6) was absent in our work.

Our experiments were performed before pure cocarboxylase was available. The chemical synthesis of cocarboxylase from thiamine (8) made it possible to test the question of activation directly.[1] The results which we have obtained demonstrate that activation of cocarboxylase by thiamine is dependent upon the type of yeast employed. When brewers' bottom yeast, which we have consistently employed in our experiments, is used as the source of

[1] We are indebted to Merck and Company, Inc., Rahway, New Jersey, for generous gifts of thiamine and synthetic cocarboxylase.

the enzyme, our previous results (2, 3, 7) may be confirmed, for little or no activation can be observed. However, in the presence of bakers' yeast atiozymase, the observations of Ochoa and Peters (6) may readily be verified. Our observations upon the behavior of the two types of yeast are in accord with those of Lipmann (9) and Westenbrink and van Dorp (10). However, our data suggest a different mechanism for the activation of cocarboxylase by thiamine from that suggested by Westenbrink and van Dorp (10).

Methods

The methods which we employ have been described previously (2, 3, 7). Two samples of brewers' bottom yeast obtained from local breweries have been studied. Three different samples of commercial bakers' yeast have also been used. All of the fresh yeast samples were crumbled finely and dried rapidly at room temperature with the aid of a fan. The dried yeast was stored in the refrigerator. Atiozymase (alkaline washed yeast) was prepared by washing these yeasts in 50 volumes of 0.1 M Na_2HPO_4 buffer at room temperature for 6 to 10 minutes. In order to eliminate the possibility that the results obtained, particularly with the brewers' yeast, were due to incomplete washing, we have varied the number of alkaline washes from two to five times. A final washing with water was always employed to remove the alkaline phosphate. The washed yeast was either suspended in buffer at pH 6.2 and used directly, or it was spread on a glass plate and dried rapidly with the aid of a fan. Dried atiozymase shows about 20 per cent loss in activity when stored at room temperature in a desiccator for a week.

Magnesium, manganese, sodium pyruvate, sodium iodoacetate, and sodium fluoride were employed in the concentrations previously described (2, 7). The experiments were conducted in either 0.1 M sodium and potassium phosphate, M/15 maleate, or M/15 succinate buffers, all at pH 6.2. In general, the metallic activators, inhibitors, coenzymes, substrate, and buffer as desired were added to the Barcroft vessel before the introduction of the atiozymase. Immediately after the addition of the yeast enzyme, the vessels were connected to the manometers, placed in the constant temperature bath, and equilibrated for 5 minutes. The

cocks were then closed and readings were taken at 10 minute intervals.

Results

A large number of experiments have been performed with atiozymase prepared from bakers' and brewers' yeast in order to determine the activating effect of thiamine upon cocarboxylase. The difference between atiozymase preparations from bakers' and brewers' yeast may readily be observed in Table I. The results of twelve experiments with brewers' yeast atiozymase under varying conditions showed an average activation of only 10 per cent upon the addition of thiamine. Eight experiments with bakers' yeast atiozymase show that the addition of thiamine to cocarboxylase causes activation to the extent of 322 per cent. The magnitude of the difference between the two types of yeast with respect to activation by thiamine readily explains the conflicting results of our work (2, 3, 7) and that of Ochoa and Peters (6).

Several interesting features of these experiments may be noted. First, it can be seen that the enzyme prepared from brewers' yeast is more active than that made from bakers' yeast. Second, the failure to achieve activation in the case of brewers' yeast cannot be due to thiamine already present in the yeast, since bacterial assay of atiozymase Preparation 4A by the method of West and Wilson (11) shows the presence of less than 0.5 γ of thiamine per gm. of atiozymase. Since only 100 mg. of enzyme are employed in these experiments, it is obvious that the amount of thiamine present is much too small to account for the failure to achieve activation. It may also be recalled that Lohmann and Schuster (1) and Hennessy and Cerecedo (12) have shown that little free thiamine is present in brewers' yeast.

On the other hand, the activation of cocarboxylase by thiamine in the presence of bakers' yeast atiozymase cannot possibly be due to the phosphorylation of the thiamine to form cocarboxylase by the enzymes present in this alkaline washed yeast. The evidence for this is quite conclusive, for Ochoa and Peters (6) have shown that pyrimidine fragments of thiamine activate cocarboxylase in a manner similar to that of the intact thiamine molecule. They have also shown that activation occurs in the presence of iodoacetic acid.

TABLE I

Relation of Bakers' and Brewers' Yeast Atiozymase to Activation of Cocarboxylase by Thiamine

1 gm. of bakers' or brewers' yeast was washed as indicated with 50 volumes of 0.1 M Na_2HPO_4 for 6 minutes each time, and then once with 50 volumes of water. For the experiments with phosphate-free buffers two or three short washings with water followed the alkaline phosphate washing. The washed yeast preparations were suspended in 10 cc. of buffer (pH 6.2) as indicated. Each Barcroft vessel contained 1 cc. of washed yeast suspension and standard quantities of sodium pyruvate, magnesium, and manganese. In addition, 1 γ of cocarboxylase, sufficient thiamine to give maximum activation (10 to 100 γ), 0.005 M sodium iodoacetate, and 0.04 M sodium fluoride were added as indicated.

Experiment No.	Yeast	No. of alkaline washes	Buffer	CO_2, c.mm. per hr.				
					Cocarboxylase	Cocarboxylase + thiamine	Iodoacetate, cocarboxylase, thiamine	Cocarboxylase, thiamine, NaF
1	Bakers', No. 1	2	PO_4		19.4	286	215	
2	" " 2	2	"		109	407	330	
3	" " 2	2	0.2 M maleate	3	94	351		505
4	" " 2	2	0.2 " succinate	5	27.7	232		
5	" " 2	2	PO_4	5	42	261		
6	" " 2	2	"	23	126	472		530
7	" " 2	3	Maleate + PO_4	0	176	469		520
8	" " 2	2	PO_4	20	65	285		
1a	Brewers', " 1	2	"	18.1	598	667	604	
2a	" " 1	2	"	13	392	392	323	
3a	" " 1	2	Maleate	13.5	550	565		
4a	" " 1	2	"	16.4	505	504		
5a	" " 1	2	PO_4	14.7	498	510	509	
6a	" " 1	2	"	96.2	542	634	608	
7a	" " 1	4	"		775	833		
8a	" " 1	4	"		550	626		
9a	" " 1	5	"		359	464		
10a	" " 1	3	"	32.8	392	524		
10b	" " 2	2	"	137	654	724		
10c	" " 2	2	"	137	765	844		

This observation has been confirmed by our work, and we have also shown that sodium fluoride does not inhibit thiamine activa-

tion (Table I). Each of these compounds effectively inhibits cocarboxylase synthesis under specific conditions (2, 3, 7). Final proof for the view that thiamine does not activate cocarboxylase by being converted into this compound may be seen from the fact that thiamine activates cocarboxylase even in the absence of inorganic phosphate. These experiments are particularly significant, for they emphasize the difference between the yeast

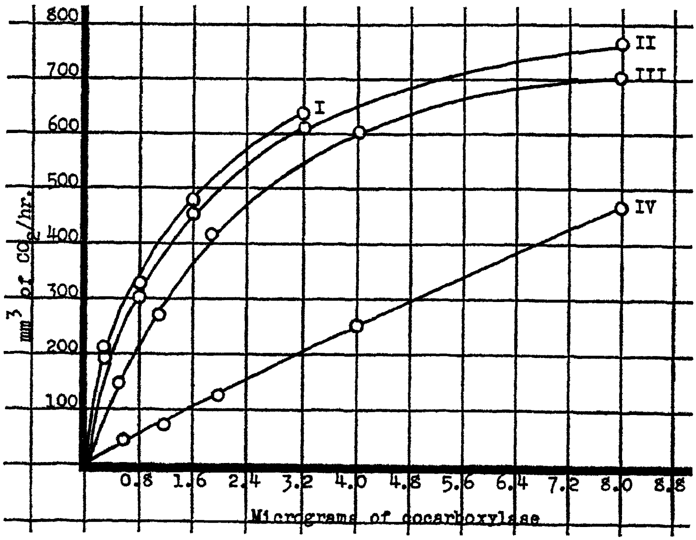

FIG. 1. The activation of cocarboxylase by 10 γ of thiamine added to different levels of cocarboxylase. Curves I and II represent brewers' yeast atiozymase with and without thiamine; Curves III and IV, bakers' yeast atiozymase with and without thiamine. The usual amounts of atiozymase, Mg, Mn, and sodium pyruvate were added. Phosphate buffer, pH 6.2, was employed.

carboxylase and the system for the oxidation of pyruvic acid by bacteria which requires inorganic phosphate (13).

We have also attempted to secure activation of cocarboxylase by thiamine at lower levels of added cocarboxylase. Fig. 1 shows the activating effect of thiamine upon atiozymase preparations from brewers' and bakers' yeasts in the presence of varying amounts of cocarboxylase. Curves I and II show that at levels between 0.4 and 3.2 γ of cocarboxylase thiamine shows no appre-

ciable activating effect with brewers' yeast atiozymase. Other unpublished experiments show no activating effect at levels as low as 0.02 γ of added cocarboxylase. Stimulation with thiamine in the presence of bakers' yeast atiozymase is clearly shown in Curves III and IV. However, even in bakers' yeast the inefficiency of thiamine activation is apparent. Thus the rates of decarboxylation of pyruvic acid are approximately equal in the presence of 4.0 γ of cocarboxylase, or 1.0 γ of cocarboxylase and 10 γ of free thiamine. Data similar to these may be found in the work of Ochoa and Peters (6). The inefficiency of thiamine activation casts some doubt upon the *in vivo* significance of this phenomenon.

Table II

Effect of Magnesium and Manganese upon Activation of Cocarboxylase by Free Thiamine in Presence of Brewers' Yeast Atiozymase

Each flask contained standard amounts of atiozymase and pyruvic acid. 1 γ of cocarboxylase, 100 γ of thiamine, 100 γ of magnesium, and 100 γ of manganese were added as indicated.

Experiment No.	Ion	CO_2, c.mm. per hr.		Per cent activation
		Cocarboxylase	Cocarboxylase + thiamine	
1	Mg	361	469	29.8
	Mn	731	815	11.3
	Mg + Mn	775	833	8.5
2	Mg	460	542	17.0
	Mn	813	891	9.6
	Mg + Mn	765	844	10.4

We have submitted a sample of our brewers' yeast to Dr. Peters and Dr. Ochoa and have received from them data which appear to be only partially in agreement with our own. Under experimental conditions which appear to be identical with our own, they have reported to us three experiments with an average activation of cocarboxylase by thiamine of 43.1 per cent. In the absence of manganese, the activity of cocarboxylase is decreased, and the stimulatory effect of thiamine rises to 106 per cent. We have performed similar experiments (Table II) and find the average activation in the presence of magnesium to be 24 per cent. In

the presence of manganese, or of magnesium and manganese, the activation was approximately 11 per cent.

It was considered possible that the differences in the behavior of our yeast in this and the Oxford laboratory might have been due to changes during shipment to England. A sample of dried yeast was therefore placed in an incubator at 37° for 2 weeks. Before storage under these conditions atiozymase prepared from the yeast showed CO_2 production of 654 c.mm. in 1 hour with 1 γ of cocarboxylase, and 724 c.mm. with 1 γ of cocarboxylase and 100 γ of thiamine. After storage atiozymase showed a value of 392 c.mm. with cocarboxylase, and 524 c.mm. with cocarboxylase and thiamine. Storage caused a decrease in the total activity of the enzyme, while it raised the thiamine activation from 11 to 34 per cent. It appears, therefore, that thiamine activation of cocarboxylase with brewers' yeast atiozymase is augmented by conditions which decrease the total activity of the enzyme system. Storage, or the omission of manganese from the system, increases the degree of activation.

Mechanism of Thiamine Activation of Cocarboxylase—The experiments already cited demonstrate that thiamine activation with alkaline washed bakers' yeast cannot be due to synthesis of the vitamin to cocarboxylase. Westenbrink and van Dorp (10) have recently suggested that there is a phosphatase present in bakers' yeast which can hydrolyze cocarboxylase. The activating effect of thiamine upon cocarboxylase, according to these workers, is due to the inhibition of the phosphatase action by one of the end-products of the hydrolytic reaction; namely, thiamine. Evidence against this view has already been presented in Table I, for thiamine activation occurs in the absence of inorganic phosphate, the other end-product of cocarboxylase hydrolysis. Both thiamine and phosphate should be required to prevent cocarboxylase hydrolysis. Still further evidence against this view is available.

Fig. 2 shows the rate of pyruvic acid decarboxylation plotted against time with different levels of cocarboxylase in the presence of bakers' yeast atiozymase. It can readily be seen that at low levels of cocarboxylase the rate of decarboxylation of pyruvic acid is practically linear. With higher levels of cocarboxylase, there is a gradual decrease in velocity, owing presumably to the accumulation of appreciable quantities of acetaldehyde, which

Lohmann and Schuster have shown to be toxic to the enzyme (1). The fact that there is linear CO_2 production with low levels of cocarboxylase argues strongly against a decrease in the amount of cocarboxylase of the system as a result of phosphatase action.

Since alkaline washed brewers' yeast responds only to cocarboxylase, and is insensitive to the addition of thiamine, it seemed that we might add cocarboxylase to alkaline washed bakers' yeast, both in the presence and absence of thiamine, and then determine the fate of this cocarboxylase by assaying the boiled bakers' yeast atiozymase with alkaline washed brewers' yeast in the usual

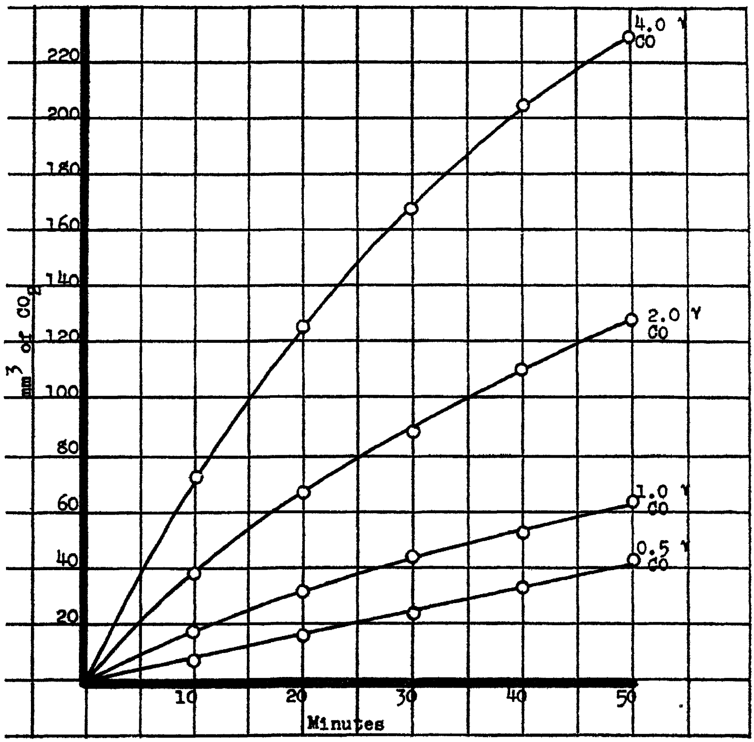

FIG. 2. The rate of pyruvic acid decarboxylation at different levels of cocarboxylase. *CO* = cocarboxylase. The usual amounts of atiozymase, Mg, Mn, and sodium pyruvate were added. Phosphate buffer, pH 6.2, was employed.

manner. We, therefore, added 1 γ of cocarboxylase, and the usual quantities of magnesium and manganese to 100 mg. of alkaline washed bakers' yeast, and after aerobic incubation at 30° for various periods of time, we boiled the yeast for 5 minutes and determined the cocarboxylase present in an aliquot, using brewers' yeast atiozymase. Similar experiments were performed with the

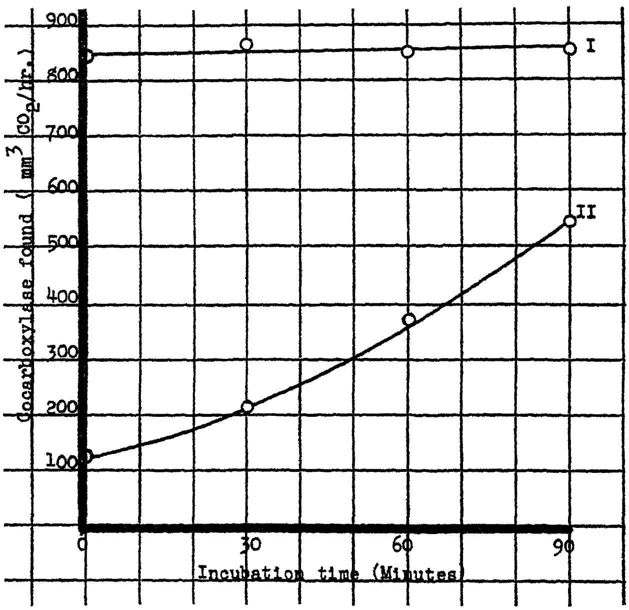

FIG. 3. The recovery of cocarboxylase incubated at 30° with alkaline washed bakers' yeast for various time periods. Curve I, 1 γ of cocarboxylase and 50 of thiamine were incubated together; Curve II, 1 γ of cocarboxylase was incubated alone. The assay was performed by adding sodium pyruvate and 100 mg. of brewers' yeast atiozymase to the boiled bakers' yeast.

addition of 1 γ of cocarboxylase and 50 γ of thiamine to the bakers' yeast enzyme. The results are shown in Fig. 3. When cocarboxylase and thiamine were incubated together with the bakers' yeast atiozymase, no change in the cocarboxylase content occurred with time. But when cocarboxylase was incubated alone with the bakers' yeast, a gradually increased recovery of cocarboxylase occurred with increasing length of incubation.

The smallest recovery of cocarboxylase was obtained when the bakers' yeast enzyme was boiled immediately after the addition of cocarboxylase. The few seconds required for this procedure seem much too short to permit enzyme action as a cause of the disappearance. Likewise, the reappearance of the cocarboxylase as a result of incubation cannot be due to resynthesis of cocarboxylase produced by immediate hydrolysis of the added cocarboxylase, because alkaline washed yeast lacks the other coenzymes necessary for cocarboxylase synthesis (2, 7).

Any explanation of these results must consider two facts; first, that the apparent rapid disappearance of cocarboxylase is prevented by thiamine; second, that the cocarboxylase reappears with time under conditions in which the reappearance cannot be due to resynthesis. It seemed most likely that these results were due to the ability of the bakers' yeast to bind cocarboxylase in such a manner that even after 5 minutes in a boiling water bath, the added cocarboxylase was not available to the brewers' yeast enzyme. Since thiamine was present in a large excess, it might prevent this binding by being itself adsorbed. The increased recovery with incubation suggested that changes in the bakers' yeast atiozymase upon incubation might be the mechanism for the release of the bound cocarboxylase.

In an effort to test this hypothesis, further experiments were devised. 1 γ of cocarboxylase was added to unboiled bakers' yeast atiozymase and to the same atiozymase which had been boiled for 7 and for 15 minutes. After incubation for 5 minutes, pyruvic acid and alkaline washed brewers' yeast were added and the rate of CO_2 production was measured. Similar experiments with cocarboxylase and thiamine added together were also performed. The results are shown in Table III. It can be seen that the ability of the bakers' yeast atiozymase to bind cocarboxylase in such a manner that the coenzyme is unavailable to the brewers' yeast enzyme is largely destroyed by 15 minutes in a boiling water bath. In the presence of thiamine, however, the cocarboxylase is available to the brewers' yeast enzyme, even when it has been added to the unboiled bakers' yeast enzyme.

Another experiment of interest in this connection is shown in Table IV. If cocarboxylase is added first to the brewers' yeast enzyme, and then unboiled bakers' yeast atiozymase is added,

there is no inhibition of decarboxylation. On the other hand, if the cocarboxylase is added first to the bakers' yeast, and then the brewers' yeast is added, there is considerable inhibition, which, as in the previous experiment, is released by the addition of thiamine in addition to cocarboxylase. This experiment also shows

Table III

Effect of Boiling upon Binding of Cocarboxylase by Bakers' Yeast Atiozymase

1 γ of cocarboxylase was added to 100 mg. of bakers' yeast atiozymase unboiled, boiled 7 minutes, and boiled 15 minutes, in the presence of Mg and Mn. After 5 minutes incubation brewers' yeast atiozymase and pyruvic acid were added. The control flask contained only brewers' yeast atiozymase and cocarboxylase.

	CO_2
	c.mm. per hr.
Control	464
Unboiled bakers' yeast atiozymase + cocarboxylase	137
Same + 50 γ thiamine	440
Bakers' yeast atiozymase boiled 7 minutes + cocarboxylase	372
Same + 50 γ thiamine	470
Bakers' yeast atiozymase boiled 15 minutes + cocarboxylase	414
Same + 50 γ thiamine	456

Table IV

Binding of Cocarboxylase by Bakers' and Brewers' Yeast Atiozymase

1 γ of cocarboxylase was added to 100 mg. of alkali-washed brewers' and bakers' yeast respectively. After 5 minutes the other yeast and pyruvic acid were added and CO_2 production was measured.

Original yeast	Yeast added at 5 min.	CO_2
		c.mm. per hr
Brewers'		298
"	Bakers'	268
Bakers'	Brewers'	118
" + 50 γ thiamine	"	288

that the phosphatase of bakers' yeast, if present, has no effect upon cocarboxylase which has been adsorbed on brewers' yeast atiozymase. The results are best explained by considering that cocarboxylase which has been adsorbed on the brewers' yeast enzyme is retained with sufficient tenacity to prevent its being affected by the addition of bakers' yeast.

DISCUSSION

Our results have demonstrated that the activation of cocarboxylase by thiamine, first noted by Ochoa and Peters (6), is dependent upon the type of yeast employed. Brewers' yeast shows little activation, while bakers' yeast exhibits a strong activation effect. The failure to achieve activation with brewers' yeast is not due to thiamine already present in the alkaline washed yeast, for bacterial analysis of the atiozymase shows insignificant amounts of thiamine to be present. Brewers' yeast can be made to show some activation by processes which decrease the total activity of the enzyme system. Such procedures include the omission of manganese, or aging the yeast at 37°.

With bakers' yeast it has been shown that the activation phenomenon is not due to synthesis of the added thiamine to cocarboxylase, for activation occurs in the absence of inorganic phosphate, in the presence of inhibitors of cocarboxylase synthesis, and, as shown by Ochoa and Peters, in the presence of fragments of the thiamine molecule. Nor can the explanation proposed by Westenbrink and van Dorp (10), that the thiamine effect is due to the inhibition by the vitamin of a phosphatase capable of splitting cocarboxylase, be considered tenable, for our experiments demonstrate that under our conditions cocarboxylase is not destroyed by bakers' yeast atiozymase. Our experiments suggest that there is a heat-labile substance present in bakers' yeast which adsorbs cocarboxylase without the production of an active enzyme. The activating effect of thiamine upon cocarboxylase appears to be due to the fact that the large excess of thiamine needed for maximum activation can saturate this material and so permit the adsorption of the cocarboxylase upon the active apoenzyme. This hypothesis is in accord with all observations upon the nature of this activation performed in this and other laboratories. Thus, it is in accord with the observation of Lipmann (9), Westenbrink and van Dorp (10), and ourselves (unpublished work) that the excess thiamine activates cocarboxylase only when it is added to the bakers' yeast enzyme before or simultaneously with the cocarboxylase. If cocarboxylase is added first, and thiamine added a few minutes later, activation does not occur.

The nature of this material is not yet known. However, the heat lability of the material suggests that it is protein. The fact

that bakers' yeast is grown under highly aerobic conditions, coupled with the observation of Greenberg and Rinehart (14) that reducing agents also activate bakers' yeast atiozymase, suggests that the substance which adsorbs the cocarboxylase might be an oxidized and therefore inactive form of the enzyme.

Our data are difficult to reconcile with those of Westenbrink and van Dorp. Experience with the variability exhibited by different types of yeast makes it possible that the activation phenomenon in the presence of English bakers' yeast is properly interpreted as being due to thiamine inhibition of cocarboxylase hydrolysis. However, the yeast employed by Westenbrink and van Dorp appears to be identical with our bakers' yeast in all other respects. A more likely explanation therefore, may be that Westenbrink and van Dorp have employed 5 times as much cocarboxylase in their experiments as we have in our work. We have chosen the low level of cocarboxylase employed in these experiments so that all of the added cocarboxylase might be adsorbed upon the enzyme surface. Experiments upon the synthesis of cocarboxylase by bakers' yeast enzymes have shown that less than 5 γ of cocarboxylase can be synthesized by 100 mg. of alkaline washed bakers' yeast. Since the degree of synthesis is limited by the amount of specific apoenzyme which binds the cocarboxylase (3, 15), it seems likely that Westenbrink and van Dorp have employed an excess of cocarboxylase. In view of the small degree of dissociation between the coenzyme and apoenzyme at pH 6.2, it seems possible that bakers' yeast phosphatase may hydrolyze free cocarboxylase but not that which is bound to the apoenzyme. The data of Westenbrink and van Dorp give no indication of the amount of cocarboxylase which has been hydrolyzed in their experiments. It may also be noted that their phosphatase is remarkably active in the absence of metallic activators like magnesium or manganese, since alkaline washed yeast contains little of these metals (1).

SUMMARY

1. The activation of cocarboxylase by thiamine has been shown to depend upon the kind of yeast employed in the test. Bakers' yeast shows this phenomenon strongly; brewers' yeast exhibits it only to a very minor degree.

2. The mechanism of thiamine activation of cocarboxylase has been investigated. It is suggested that there is a heat-labile material present in bakers' yeast which can adsorb cocarboxylase without the production of an active enzyme. The addition of excess thiamine saturates this material and thus permits the adsorption of the cocarboxylase upon the active apoenzyme.

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THE POLAROGRAPHIC DETERMINATION OF KETONIC STEROIDS

By JOHN K. WOLFE,* E. B. HERSHBERG,† and LOUIS F. FIESER

(*From the Converse Memorial Laboratory, Harvard University, Cambridge*)

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From available data concerning the scope of Heyrovsky's polarographic method of analysis[1] it would appear that in the field of the sex hormones any application of the method in its present form probably would be limited to the study of those ketonic steroids having an ethylenic double bond in conjugation with the carbonyl group. To produce a characteristic cathodic wave, an organic compound must possess one or more functional groups rendering it capable of undergoing electroreduction at the dropping mercury electrode at a potential within the range limited by the discharge potential of the most readily reduced ion of the electrolyte mixture employed to carry the diffusion current and to provide buffer action. The only hormones offering any possibility for polarographic reduction are those which are ketonic, and studies of simple ketones by Winkel and Proske (4) and by Adkins and Cox (5) have shown that reduction of the carbonyl group occurs in the measurable range of potential only if this group is activated by an adjacent double bond, as in the α,β-unsaturated ketones with the system $>\!C{=}\underset{|}{C}{-}\underset{|}{C}{=}O$. This structural characteristic is found in testosterone, desoxycorticosterone, and progesterone, and in view of the importance of these hormones and the interest in their determination the application of the polarograph to the

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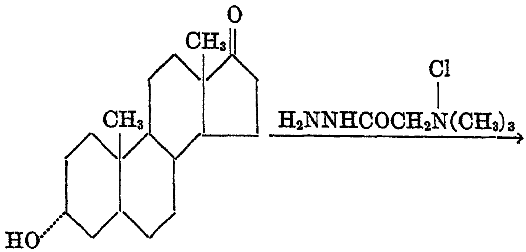
† Research Fellow on grants from the National Cancer Institute and Eli Lilly and Company.

[1] For references, and for discussions of the principles of the polarographic method and its applications in organic chemistry, see the review papers by Kolthoff and Lingane (1), Müller (2), and Winkel and Proske (3).

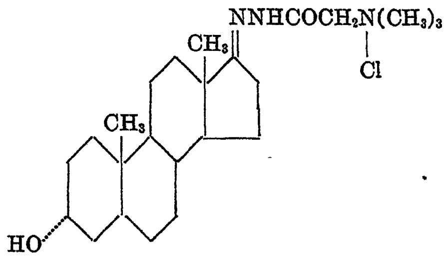
hormone field would seem worth while even if limited to this type of substance.

Before installation of the apparatus required for the present work, preliminary trials kindly carried out by Professor H. Adkins and Dr. R. H. Baker had definitely established that the Δ^4-unsaturated 3-ketosteroids are indeed determinable with the use of the dropping mercury electrode. Since then a paper by Eisenbrand and Picher (6) has appeared presenting full data on this point and showing that the saturated ketonic hormones are indifferent and do not interfere with the determination of the α,β-unsaturated ketones. While some additional observations on the behavior of these compounds will be recorded below, the principal outcome of this investigation is the development of a method for the polarographic characterization of saturated as well as unsaturated ketones of the hormone series. It occurred to us that such substances might be brought within the measurable range in one of two ways. Either the $\rangle C{=}N{-}$ linkage resulting from a condensation with a suitable amine derivative might prove more readily reducible than the original $\rangle C{=}O$ group, or a reagent might be found, capable of fixation to either the carbonyl or the alcoholic group of the hormone, having a second functional group to lend adequate reducibility to the derivative as a whole. The initial trial prompted by the first consideration proved so promising that work along the second line has been deferred for the present.

The nitrogen derivative found satisfactory is obtained by condensation of the ketone with Girard's reagent (7), more specifically Reagent T (trimethylacethydrazide ammonium chloride). Thus androsterone reacts very rapidly with the reagent to give a derivative having an ionic ammonium salt group which renders the substance soluble in water. The reagent has been used extensively for the separation of ketonic from non-ketonic steroids in the course of the isolation of hormones from urinary extracts (7) and from preparative reaction mixtures (8), and also in a refinement and extension of the colorimetric assay of urinary androgens (9). While androsterone itself is completely indifferent to the polarographic diffusion current in the range studied, the Girard derivative, when investigated in an aqueous buffer solution under suitable conditions, gives a well defined polarographic curve or



Androsterone



Girard derivative

wave. Fortunately the Girard reagent itself discharges at such a distinctly higher level (more negative potential) that the presence of uncombined reagent does not inferfere with the polarographic characterization of the condensation product. Thus an extract of unknown hormone content can be prepared for polarographic analysis very easily and rapidly by short heating with an excess of Girard's reagent in acetic acid, and subsequent addition of ammonium chloride and of enough sodium hydroxide to give a sodium acetate-acetic acid buffer of the required pH.

The method of polarographing the Girard derivative has been found applicable to a number of ketonic steroids, and interesting indications of specificity of analysis have been encountered in the course of exploring the possibilities for the utilization of the method in determining hormones in urinary or tissue extracts. In this paper the applicability of the method is illustrated by its use in the determination of urinary androgens.

Apparatus

The electrical equipment consisted of a Leeds and Northrup Electro-Chemograph, including a polarizing unit, an electronic amplifier, and a recording potentiometer (Micromax), which draws

a line in ink constituting a permanent record of the polarogram. For convenience in operating and reading the dials of the polarizer, this was mounted on its side in a slightly tilted wooden casing which also served to support an auxiliary plug type resistance box housing the external shunts furnished with the apparatus. The motors driving the recorder chart and the slide wire mechanism of the polarizer were synchronized to a chart speed of 36 inches per hour. In our experiments the following different shunts were used to vary the galvanometer deflection and provide different sensitivities: A, 10 scale divisions (2.54 cm.) = 4 microamperes; B, 10 scale divisions = 2 microamperes; C, 10 scale divisions = 1 microampere; D, 10 scale divisions = 0.5 microampere.

The ordinates of the recorder curves or polarograms represent the applied potential, each division being equivalent to 0.1 volt. The abscissas, with 100 divisions for the scale deflection of 10 inches, indicate the diffusion current, to be read in terms of a given number of microamperes per division according to the shunt used. The resistance box mounted as described above provided a convenient arrangement for the necessary frequent interchange of shunts.

The assembly of the dropping mercury electrode and cell adopted for the present work after trial of a number of other types is shown in Fig. 1. A leveling bulb, constituting a mercury reservoir and provided with a platinum wire supported through the opening for establishing electrical contact through a clip connector and a copper cable, was connected by means of a sulfur-free gum rubber tubing to a burette form tube calibrated in mm. and serving for the reading of the mercury level. Connection to the reservoir was made through a side tube sealed on above the stop-cock. The stop-cock was of the grease-free type (Nolub, 1 mm. bore, Scientific Glass Apparatus Company) and its plug was secured against the mercury pressure by means of a pair of interlocking brass plates held by rubber bands. With a stop-cock at this point the mercury can be held in reserve until a measurement is to be made. When the burette is first filled, the stop-cock is loosened enough to allow a film of mercury to spread over the ground surfaces and act as a lubricant. In operation, mercury is allowed to flow at a rate of 1½ seconds per drop. The capillary tip for the delivery of drops of mercury to the cell was attached

to the burette tube by means of a standard taper 10/18 ground joint held in place by rubber bands attached to a hooked wire loop around the lower joint and extending over the stop-cock.

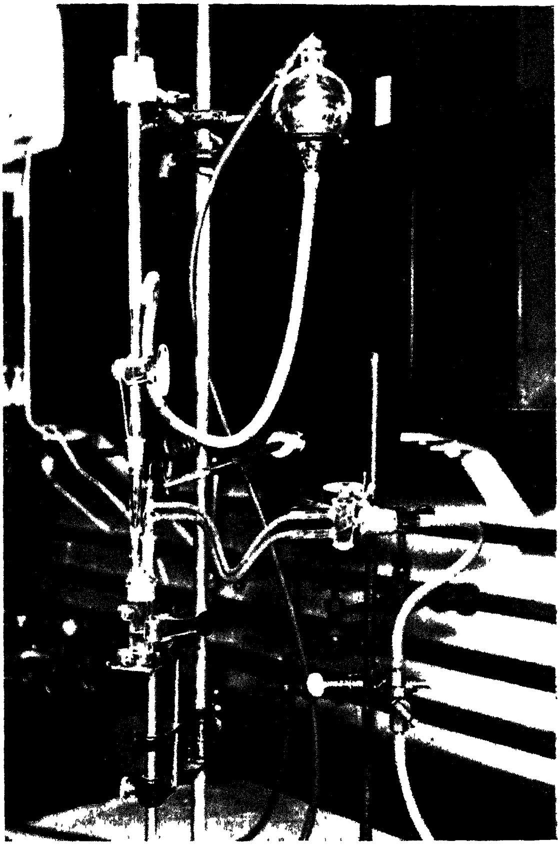


FIG. 1. Dropping mercury electrode and cell assembly

Details of the construction of the cell are given in Fig. 2. The cell was made from a weighing bottle with a ground glass cap of standard commercial specifications designated as "height 50 mm., inside diameter 15 mm., standard taper 19/10" (Scientific Glass Apparatus Company; see the actual dimensions given in Fig. 2). The ground glass cap was fitted with one opening for the loose

insertion of the tip of the dropping mercury electrode and with another, consisting in a standard taper joint, to accommodate a tube extending to the bottom of the cell and constituting a nitrogen inlet tube as well as providing for electrical connection to the mercury anode. The platinum wire to make contact with the pool of mercury in the cell was electrically spot-welded to a tungsten rod carrying a welded-on copper cable[2] to which electrical connection was made with a spring clip. Several caps and elec-

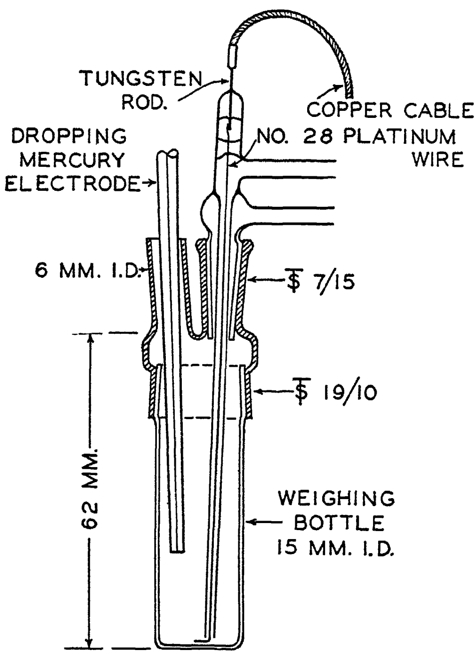


FIG. 2. Construction of the cell

trode assemblies were constructed and used interchangeably with the commercially available weighing bottles. The tube carrying the anode wire was provided with two inlets for nitrogen, one for deaerating the solution prior to a measurement and the other for passing nitrogen over the solution during a measurement or during

[2] A suitable tungsten-copper union may be obtained from the Callite Products Company, Union City, New York, under the specification 0.030 inch (0.76 mm.) Kulgrid C stranded tungsten weld.

any period subsequent to deaeration when protection from air was desired without undue evaporation. The nitrogen could be directed to either inlet by manipulation of a 3-way stop-cock. The gas taken from a high pressure cylinder of prepurified nitrogen was led through two wash bottles containing Fieser's solution (10), one charged with lead acetate solution, and one of water. A slight pressure was maintained in the train and the flow of purified nitrogen to the cell was regulated by means of a small needle valve.

Reagents and Hormone Samples—

Mercury. C.P. reagent quality mercury was used without further purification.

Water. Distilled water was redistilled in an all-glass apparatus.

Isopropanol. Eastman Kodak Company material was carefully distilled through a 1 meter column and the fraction boiling at 82.3–82.5° selected.

Acetic acid. This was purified by redistillation from potassium permanganate.

Tetraethylammonium hydroxide. The reagent was prepared from tetraethylammonium bromide and silver oxide by the procedure of Peracchio and Meloche (11).

Girard's reagent (T) was prepared by the method of Girard (7, 12) and purified by two recrystallizations from absolute alcohol. Material which has been stored for several months should be recrystallized before use.

The melting points and sources of the hormone samples used are indicated in Table I. We are greatly indebted to Dr. O. Wintersteiner for the samples of rare cortical steroids, to Dr. Erwin Schwenk of the Schering Corporation for a generous supply of pregnenolone, and to Dr. Talbot and Dr. Friedgood for the use of samples obtained for colorimetric studies.

Desoxycorticosterone, m.p. 139–141°, corrected, was obtained by hydrolysis of the acetate according to Reichstein and von Euw (13). Standard methods were employed for the preparation of cholestanone (14), m.p. 128–129°, corrected, cholestenone (15), m.p. 78.5–80.5°, corrected, and coprostanone (16, 17), m.p. 60.5–61.5°, corrected, except that in the last case palladium-barium sulfate was employed as the hydrogenation catalyst. A sample of pure Δ^1-cholestenone hydrate, m.p. 107–108°, corrected, was kindly supplied by Dr. R. P. Jacobsen (18).

Girard Derivative of Cholestanone (*Cholestanone Betaine Hydrazone Chloride*)—A suspension of 0.2 gm. of cholestanone and 0.087 gm. of Girard's reagent (T) in 0.5 cc. of glacial acetic acid was warmed for 10 minutes on the steam bath and the resulting solution was evaporated to dryness under reduced pressure at 100°. The solid residue on three crystallizations from methanol-

TABLE I

Source and Properties of Hormone Preparations

Compound	M.p., corrected	Source
	°C.	
Androsterone	183–184	Ciba Pharmaceutical Products, Inc.*
Isoandrosterone	172–173	" " †
Testosterone	153–154	" " †
Dehydroisoandrosterone	149–151	Schering Corporation†
Desoxycorticosterone acetate	159–160	" " *
Progesterone	126–127	" " *
Estrone	257–258	" " †
Δ^5-Pregnenol-3-one-20	184–186.5	" "
Corticosterone	182	Dr. O. Wintersteiner
17-Hydroxydehydrocorticosterone (Δ^4-pregnenediol-17,21-trione-3,11,20; Substance F)	208	" " "

* Supplied to Dr. N. B. Talbot.
† Supplied to Dr. H. B. Friedgood.

acetone afforded colorless microcrystals of the monohydrate melting at 233–234°, corrected, with decomposition.

Analysis—$C_{31}H_{56}ON_3Cl\cdot H_2O$. Calculated. C 68.91, H 10.83
Found. " 68.38, " 10.83

Dehydroisoandrosterone p-Nitrophenylhydrazone Acetate—A mixture of 0.1 gm. of dehydroisoandrosterone acetate, 0.5 gm. of *p*-nitrophenylhydrazine, and 0.5 cc. of glacial acetic acid was warmed at 100° for 5 minutes and the resulting stiff paste was stirred with 5 cc. of alcohol and the light yellow, crystalline product collected and washed with alcohol. On crystallization from a rather large volume of isopropanol the substance formed fine yellow needles, melting with decomposition at 291–292°, corrected.

Analysis—$C_{27}H_{35}O_4N_3$. Calculated. C 69.65, H 7.58
Found. " 69.55, " 7.57

Investigation of 17-Ketosteroids

The procedure detailed below for the preparation and polarographic analysis of a solution of the Girard derivative of a ketone was developed after numerous trials of a largely empirical nature and on the basis of certain observations and considerations which will be brought out in the subsequent discussion. This procedure was worked out with particular reference to the determination of urinary androgens and companion ketones.

Standard Procedure—A suitable volume of a known solution of a pure hormone (usually 1 mg.) in isopropanol,[3] or a volume of alcoholic extract containing the non-phenolic hormone fraction equivalent to 50 cc. of urine, is placed in a 12 × 75 mm. Pyrex test-tube and evaporated to dryness under diminished pressure (water pump vacuum). The process can be conducted without bumping of the liquid by first allowing the solution to become chilled by evaporation at room temperature and then warming the tube gently in a water bath while the liquid is rocked to and fro. The usually oily residue is treated with 0.05 cc. of a fresh solution of 100 mg. of Girard's reagent in 1 cc. of glacial acetic acid,[4] measured with a 0.10 cc. pipette, and the test-tube is rotated to insure mixing. The tube is then closed with a cork stopper, immersed in a water or steam bath at 100°, and heated for 2 minutes. The sample is then cooled, diluted with 0.95 cc. of purified water, and thoroughly mixed. A 0.25 cc. portion[5] of the solution is measured promptly with a 1 cc. pipette into the cell, followed by 0.50 cc. of 0.50 N ammonium chloride solution and 0.625 cc. of 0.20 N sodium hydroxide solution. The solution is made up to

[3] In the case of the rarer specimens a 1 to 2 mg. sample was weighed accurately to 1 to 5 γ on a microbalance and treated with sufficient measured solvent to give a solution of 2 mg. per cc. By employing isopropanol as solvent, the same solutions could be used for polarographic analysis in the absence of Girard's reagent.

[4] It is recommended that the solution be made up daily, or as needed. Satisfactory determinations have been made with 2 day-old solutions, but deterioration was evident after longer periods of storage.

[5] The remaining 0.75 cc. of aqueous solution of the Girard derivative is kept in reserve for a time in case further determinations become desirable. These should be made within an hour after the dilution with water, for the solution may deteriorate and become cloudy if kept for longer periods. The mixture is more stable before it has been diluted, and samples may be brought to this stage and safely kept for as long as 6 hours before dilution and subsequent analysis.

a volume of 2.50 cc. by the addition of 1.125 cc. of water and is then ready for polarographic analysis.

Sufficient mercury is added to cover the bottom of the cell and make contact with the platinum wire. With the electrical connections completed, a polarizing potential of -1.0 volt is applied, with the galvanometer Sensitivity D, and a stream of nitrogen is bubbled through the solution. The galvanometer at first shows a considerable displacement to the right of the initial zero point, owing to the oxygen present, but the pointer soon recedes as this is removed (about 5 minutes). As soon as the galvanometer reading has become constant, the nitrogen stream is by-passed over the liquid and the solution is polarized at a potential increasing from -1.0 to -1.8 volts and at whatever sensitivity is judged to be the most promising for initial trial. It may be necessary to investigate all four sensitivities in order to select the one giving the best defined and most accurately measurable wave, preferably covering a span of from 10 to 40 mm. With some practice a satisfactory result usually can be obtained by making polarograms at no more than two different sensitivities. Occasionally it is found necessary to add a drop or two of 0.05 per cent aqueous glue solution to overcome a current surge at the terminal discharge.

The quantity of Girard's reagent specified calls for further comment. The amount used (5 mg.) in preparing the derivative is about 6 times that theoretically required to condense with the maximum quantity of hormone which the procedure is designed to accommodate (*e.g.* 1 mg. of androsterone), but experimentation has shown that this large excess is essential. In trials conducted with 5 mg. of reagent and varying amounts of hormone, it was found that a linear or approximately linear relationship existed between the polarographic wave span and the weight of hormone as this was increased from the lower limit to 1.2 mg., but that beyond this point added amounts produced no further effect. To provide some margin of safety, we therefore selected 5 mg. of reagent as a suitable amount to employ with a maximum of 1 mg. of hormone. If, in the analysis of an unknown sample, the amount indicated to be present exceeds the 1 mg. limit, the sample solution should be diluted and a fresh determination made.

The Girard Reagent P (pyridine) was tried but found inapplicable. The reagent itself discharges at a potential too low to

permit characterization of the ketonic condensation product. The *p*-nitrophenylhydrazone of dehydroisoandrosterone was found to give a well defined polarographic wave, but conditions for the practical application of this type of derivative to hormone analysis have not been investigated. The determination was made with 0.5 mg. of substance in 2.50 cc. of isopropanol containing 10 mg. of lithium chloride. The half wave potential was −0.92 volt and the wave span (see below) at Sensitivity D was 90 mm.

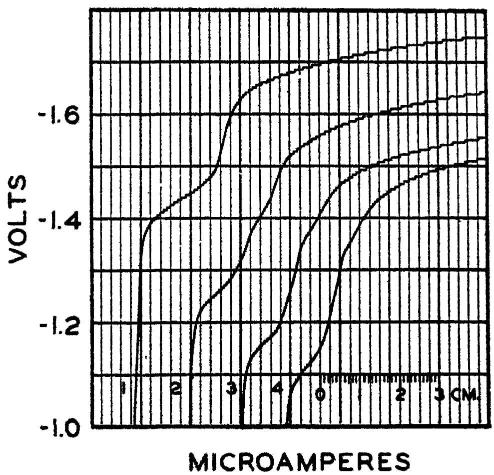


FIG. 3. Dehydroisoandrosterone-Girard derivative; effect of varying the acidity. Each of the four solutions contained a 0.25 cc. aliquot of the solution of derivative prepared from 0.42 mg. of hormone, 0.5 cc. of 0.5 N NH_4Cl, and the following volumes of additional reagents: Curve 1 (standard procedure), 1.125 cc. of H_2O, 0.625 cc. of 0.2 N NaOH; Curve 2, 1.75 cc. of H_2O; Curve 3, 1.50 cc. of H_2O, 0.25 cc. of 0.1 N HCl; Curve 4, 1.25 cc. of H_2O, 0.50 cc. of 0.1 N HCl.

A typical polarogram obtained by the standard procedure is shown in Curve 1 of Fig. 3, which is a reproduction of the actual chart drawn by the recording potentiometer. When the applied voltage is automatically increased, the diffusion current remains but little altered (nearly vertical line) until a potential is reached at which a discharge due to the Girard derivative begins to occur. After the completion of this discharge and formation of the characteristic wave, the curve again rises sharply at a near vertical

slope until a level is reached at which discharge of ionic electrolytes, including the excess Girard's reagent, occurs. Curve 1 presents a well defined wave and it is to be contrasted with Curves 2, 3, and 4, which illustrate the disadvantageous effect of progressively increasing the acidity of the solution. Solutions more alkaline than that of the standard procedure were tried with unpromising results, for although the polarograms were satisfactory with respect to form of curve there was a distinct lack of proportionality between the weight of hormone and the polarographic effect observed.

Analysis of Polarograms—Several different methods of measuring polarograms of organic compounds have been defined and discussed by Borcherdt, Meloche, and Adkins (19), but none of these methods seems applicable to the case at hand because of a significant difference in the character of the wave, a difference which is particularly apparent in analysis of urine extracts. The distinguishing characteristic is apparent even in polarograms obtained with pure hormones, as in Curve 1, Fig. 3. It will be observed that the two parts of the curve which are most nearly vertical are definitely non-parallel, and with urine extracts the divergence often is much more pronounced. This feature introduces a complication in the selection of the current range over which the discharge is attributable to the substance under investigation. In polarograms obtained with some instruments the current is plotted along the ordinate of the chart, and in this case the range covered is appropriately called the wave height (19). With the Leeds and Northrup instrument, where the alternate scheme of charting is used, the equivalent term wave-length seems ill advised because of its use in spectroscopy and we prefer the designation wave span, which is applicable to both systems. After carefully considering the problem of measuring the present wave spans from a practical and purely empirical point of view, we have adopted a method which can be illustrated with reference to Fig. 4. A line is drawn extending the initial straight and nearly vertical portion of the curve, and an estimate is made of the inflection point of the near vertical part of the curve representing the termination of the wave proper. This usually is at the mid-point of a straight line section and can be further defined as the point at which the oscillation tracings are at a minimum. A line drawn from this point perpendicular to the above extension line is taken

as a measure of the wave span. It will be seen from the typical example of Fig. 4 that the wave spans A, B, and C found by this method for a given solution polarographed at three different sensitivities bear a relationship to one another which corresponds closely to the ratio of the sensitivities; namely, 1:2:4.

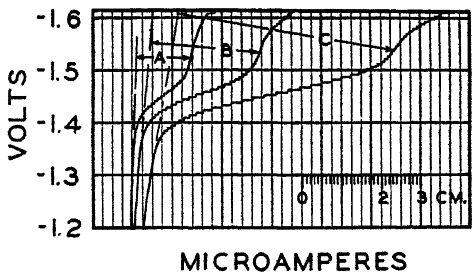


FIG. 4. Urine extract (PBBH-78-C, 0.2 cc.) with added androsterone (0.5 mg.) polarographed at three sensitivities. Sensitivities and corresponding wave spans: Sensitivity A, 13.2 mm.; Sensitivity B, 27.4 = 2 × 13.7 mm.; Sensitivity C, 54.7 = 4 × 13.7 mm.

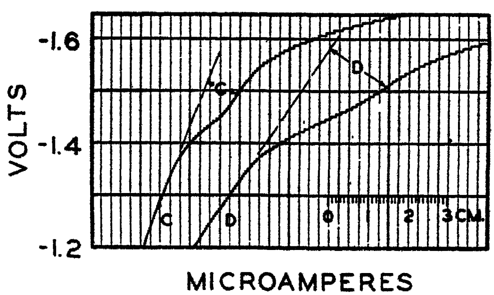


FIG. 5. Crude urine Extract CH-125-C. Sensitivity C, wave span 8.6 mm., indicating 0.105 mg. of hormone in the original sample; Sensitivity D, 17.2 mm., 0.11 mg. of hormone.

The polarographic waves obtained with urine extracts usually are less sharply defined than those given by pure hormones or by extracts containing added hormone, but the above method of analysis nevertheless appears applicable. A particularly unfavorable case is illustrated in Fig. 5, which refers to a highly pigmented neutral urine extract containing a considerable amount of nonketonic material. Although neither curve provides a very precise

basis for estimation, a reading can be made even in the less propitious case (Sensitivity D) and the results of the two observations are in good agreement. Fig. 6 shows the results obtained with a portion of the same extract after it had been put through the Girard separation to remove the non-ketonic material (9), and if this is compared with Fig. 5 it is seen that the purification results in a marked improvement in definition of the curves, the wave span being easily measured at both sensitivities. It is also noteworthy that the amount of ketonic hormone found is substantially the same as in the determinations made in the presence of the interfering non-ketonic materials. In general, it appears possible to conduct satisfactory polarographic analyses of crude

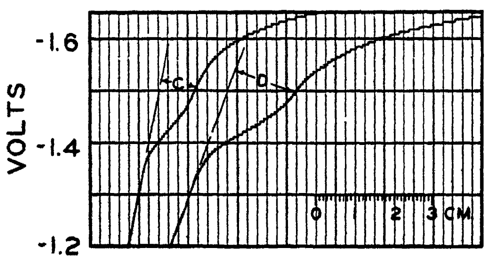


FIG. 6. Same urine extract after removal of non-ketones (Extract CH-125-K). Sensitivity C, wave span 8.1 mm., equivalent to 0.10 mg. of hormone; Sensitivity D, 16.0 mm., 0.10 mg. of hormone.

urine extracts which have not been processed by removal of the non-ketonic fraction, provided that the sample has been clarified by extraction with alkaline hydrosulfite solution (20).

Calibration Curves—In testing the method of polarographing the Girard derivatives for its applicability to problems of analysis, we first investigated the weight-wave span relationship for three pure hormones over a 10-fold range of sample weight. Alcoholic solutions of androsterone, isoandrosterone, and dehydroisoandrosterone were prepared containing 2.00 mg. per cc., and suitable portions were pipetted and polarographed by the standard Girard procedure at four sensitivities, with the results shown graphically in Fig. 7. While the relationship appears to be linear, or nearly so, in the lower concentrations, the curves diverge considerably from

the initial straight line slope as the amount of hormone is increased. The effect evidently is associated in some way with the decreasing ratio of hormone to excess Girard's reagent, and as noted above the correlation of wave span to amount of hormone breaks down entirely on increasing the weight of the latter to 1.2 mg. The

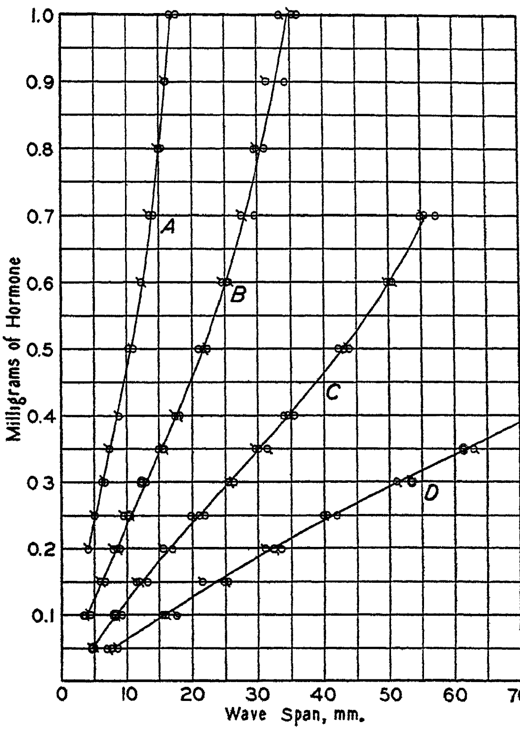


FIG. 7. Relation of polarographic wave span to weight of pure crystalline hormone (total weight of material taken for condensation with 5 mg. of Girard's reagent; 4 times that actually polarographed). Sensitivities, A, B, C, D, as indicated; O androsterone; Q isoandrosterone; Ɔ dehydroisoandrosterone.

curves given in the figure can be used as calibration graphs with reasonable assurance as to accuracy if the readings are limited to the lower ranges of concentration. Perhaps the most significant feature of the results as a whole is the demonstration that three pure steroids of the saturated 17-keto type and of essentially

the same molecular weight give exactly the same polarographic response.

The next step was to investigate the behavior of hormones in the presence of the companion substances found in urinary extracts. For this purpose small amounts of non-phenolic urine extracts were added to known samples of pure hormones and polarograms were made by the standard procedure. The ketosteroid content of the extract added was determined with the use of the calibration curves of Fig. 7, at such a dilution as to utilize only the lower extremities of the curves. Any error associated with that part of the total hormone derived from the extract was further minimized by keeping the amount added at a low level (*e.g.* 0.1 mg.). The results are recorded in Fig. 8, and it will be seen that the wave span is directly proportional to the amount of hormone and that the linear relationship is maintained accurately over the entire range of 0.1 to 1.0 mg. The situation is much more favorable than is encountered with pure hormones alone, evidently because of a stabilizing or other beneficial influence exerted by constituents of the urine extracts. This influence is manifested with no more than a trace of added extract; for example, with only 10 γ of evaporated urine extract per mg. of crystalline hormone. The effect appears to be very general, for urine extracts of a wide variety of types were used in constructing the curves and the effect was observed not only with total crude neutral fractions but with ketonic and non-ketonic fractions, separated with the use of the Girard reagent. Since non-ketonic material is probably carried in small amounts into the ketonic fraction, a trace of a non-ketone may be responsible for the "stabilization."

In Fig. 8 the ten points on the curve for the highest sensitivity (D) were all determined in special calibration runs conducted as described, with a very minute amount of urinary hormone present. Similar determinations in like number were used to define the other three curves, but here the chart includes as well the results of a number of subsequent determinations made by establishing the hormone content of a given urine extract by reference to the calibration, adding a known amount of pure hormone, and making a determination of the total hormone content. The points obtained in this way conform to the curves nearly as well as the actual calibration points and have been included on Fig. 8 to give

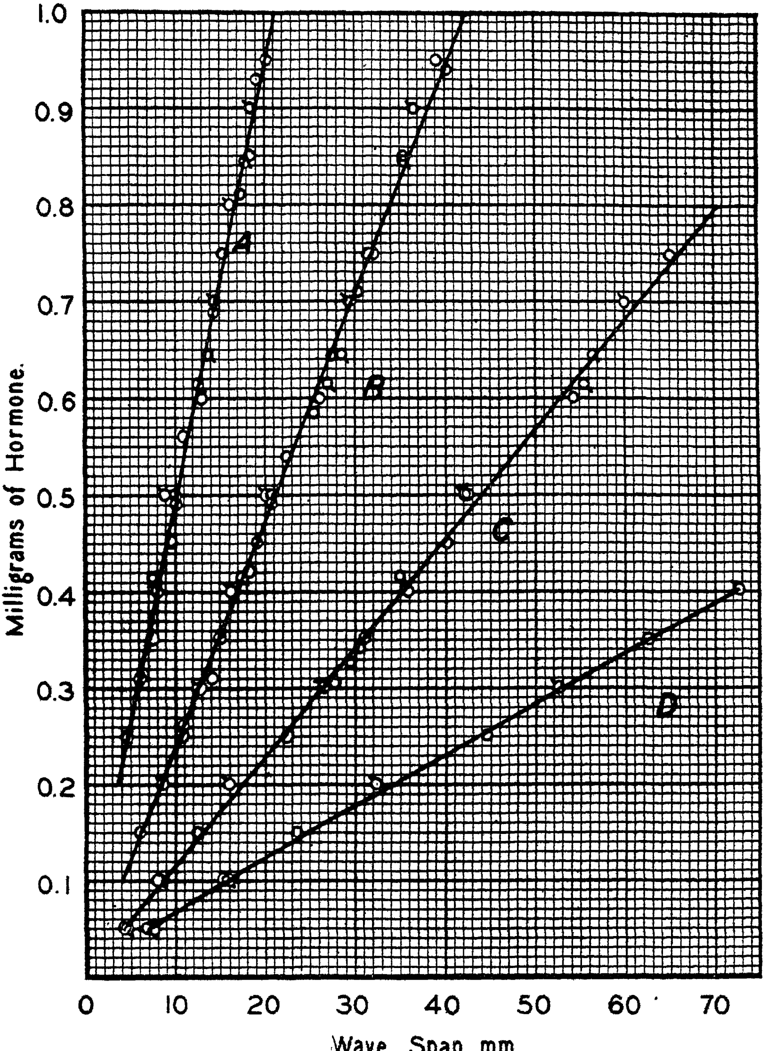


FIG. 8. Calibration curves for the determination of androgens in urine extracts. The 17-ketosteroid content, calculated as androsterone, refers to the total amount in the sample processed by the standard procedure. O urine extract + androsterone; Q urine extract + isoandrosterone; Ծ urine extract + dehydroisoandrosterone.

an indication of the magnitude of the deviations to be expected in analytical practice.

The calibration curves provide adequately for the accurate determination of the hormones over the rather wide range of from 0.05 to 1.0 mg. of material in the total sample. It should be noted that only one-fourth of the sample is taken for an actual determination and that the limit of accurate analysis by the present procedure is therefore about 12 γ. The sensitivity could be extended easily by employing a smaller electrolysis cell, but for most purposes when minute amounts are to be detected it would be necessary merely to evaporate a larger volume of urine extract.

Determination of 17-Ketones in Androgen Fraction of Urine—As a test of the generality of application of the method, analyses were made of a number of neutral urine extracts obtained from the assay laboratories of three Boston hospitals, where they had been prepared by solvent extraction of urine, removal of the phenolic material, and such other processing as is currently conducted in the routine assays of the respective laboratories. We are greatly indebted to Dr. Fuller Albright for samples from the Massachusetts General Hospital, to Dr. H. B. Friedgood for those from the Peter Bent Brigham Hospital, and to Dr. N. B. Talbot for those from the Children's Hospital. Data were kindly supplied giving the results of colorimetric assays obtained by the Zimmermann method (21) as modified by Callow, Callow, and Emmens (22), and as further elaborated in the three laboratories. The nature of procedures used is indicated in publications by Friedgood and Whidden (23) and by Talbot and associates (9, 24, 25).

The results are recorded in Table II. A given volume of the alcoholic extract (second column) was evaporated to dryness, put through the standard procedure, and the amount of hormone present in the indicated volume of extract was determined from polarograms taken at appropriate sensitivities and by reference to Fig. 8. The inclusion of two sets of figures for a given sensitivity indicates that entirely independent duplicate determinations were made with separate portions of the original extract. The agreement between the duplicates is invariably good, as is the correspondence of the determinations made at different sensitivities. The weight of hormone found is occasionally given to three decimal places merely for the purpose of indicating the magnitude of the

deviations and to illustrate the extent to which errors in measuring the wave span may influence the result in terms of mg. of hormone.

The polarographic results show a general correspondence with the results of the Zimmermann assay. That the values reported from the Massachusetts General Hospital are lower than ours is to be discounted because of a large correction factor applied in obtaining these Zimmermann values. The other colorimetric assays all tend to run significantly higher than ours. Thus in the extensive series of results supplied by Dr. Talbot, most of which refer to determinations on the separated ketonic fraction, the indicated hormone content is irregularly higher (average, about 50 γ) than that found polarographically.

The samples studied included urinary extracts from normal males and females, children, a female with an adrenal tumor, and patients with Addison's disease and in various other pathological conditions. The procedures for the dilution of the evaporated urine extract varied considerably and there is no simple or regular relationship between the volume of extract taken for analysis and the amount of urine which it represents. As an indication of the extent of variety in the samples, it may be said that they ranged from a ketonic hormone content of 1.7 mg. per liter (Extract CH-44-K) to 141 mg. per liter (Extract PBBH-105-C). Since no significant difficulties were encountered, it would appear that the polarographic method is very generally applicable to the determination of urinary androgens. It may be noted that the method is rapid and that a single worker can make as many as thirty determinations in a day. Proper processing of the extract, including a hydrosulfite wash (20), is desirable for best definition of the curve, and standardization of the amount and dilution of the extract makes for convenience of determination. On the basis of our experiences with the above samples and of certain observations by Dr. N. B. Talbot, we suggest that extracts for polarographic analysis be prepared by the following general *extraction procedure.* A 1 liter sample of urine bearing a known ratio to the daily output is extracted with solvent. The estrogen fraction is removed from a solution of the extract by four washings with 2.5 N sodium hydroxide, and the neutral fraction is washed twice with 2.5 N sodium hydroxide containing 10 per cent of sodium hydrosulfite, each time with vigorous shaking for 5 minutes. The

TABLE II

Polarographic Analysis of Neutral Urine Extracts

Urine extract*	Volume taken for analysis	Sensitivity	Wave span	Hormone found	Hormone found by Zimmermann assay
	cc.		mm.	mg.	mg.
MGH-1734-C	2.0	A	8.2	0.40	0.30
		B	16.5	0.39	
MGH-1727-C	0.5	"	18.2,17.1	0.43,0.40	0.31
MGH-1724-C	0.5	A	8.5	0.42	0.32
		B	19.6,19.2	0.45,0.45	
		C	37.4	0.43	
MGH-1737-C	0.5	B	10.5	0.25	0.28
PBBH-78-C	0.2	"	4.5	0.11	0.12
		C	8.8	0.10	
		D	17.1	0.11	
PBBH-78c-C	0.5	B	13.9,12.1	0.32,0.29	0.31
PBBH-78b-C	0.5	"	12.8,12.5	0.30,0.29	0.31
PBBH-105b-C	0.1	C	15.6,16.0	0.17_8,0.18_2	0.27
PBBH-105c-C	0.05	B	3.7	0.09_0	0.13
		C	7.4	0.08_5	
		D	15.4	0.09_5	
CH-128-K	0.2	C	7.9,7.8	0.09,0.09	
CH-127-K	0.2	"	12.2,12.1	0.14,0.14	
CH-128-C	0.2	B	4.6	0.11	
		C	9.1	0.10_5	
CH-126-C	0.2	"	9.4,9.4	0.11,0.11	0.13
		D	18.4,19.0	0.11_5,0.12	
CH-126-K	0.2	C	8.1	0.09_3	0.12
		D	15.9,15.1	0.10,0.09_5	
CH-125-C	0.2	C	9.6,8.6	0.11,0.10_5	0.14
		D	17.2	0.11	
CH-125-K	0.2	C	8.1	0.10	0.12
		D	16.0,15.4	0.10,0.09_8	
CH-1a-K	0.2	C	8.7	0.10	0.14
CH-1b-K	0.4	A	7.8	0.38	0.52
		B	15.4	0.36	
CH-1c-K	0.2	"	2.5	0.06_0	0.09
		C	4.9	0.05_5	
CH-1d-K	0.2	B	8.9	0.21	0.25
		C	18.5	0.21_5	
CH-1e-K	0.4	B	10.3	0.24	0.32

TABLE II—*Concluded*

Urine extract*	Volume taken for analysis	Sensitivity	Wave span	Hormone found	Hormone found by Zimmermann assay
	cc.		*mm.*	*mg.*	*mg.*
CH-1f-K	0.4	A	15.5	0.73	0.76
		B	31.4	0.74	
CH-21-K	0.4	A	7.3	0.36	0.35
		B	14.5	0.34	
CH-22o-K	0.5	"	12.9	0.30	0.40
CH-22n-K	0.5	"	13.4,13.6	0.31,0.32	0.38
CH-23o-K	0.5	C	36.6,33.9	0.41_5,0.39	0.44
CH-23n-K	0.5	B	18.9,17.9	0.44,0.42	0.44
CH-24-K	0.5	"	22.0,23.1	0.52,0.54	0.54
		C	45.1	0.51	
CH-27-K	0.5	"	27.0,26.6	0.31,0.30_5	0.35
CH-31-K	0.5	B	15.0,15.1	0.35,0.35	0.34
CH-32-K	0.5	A	12.6	0.60	0.75
		B	25.9,24.5	0.61,0.58	
		C	51.0	0.58	
CH-33-K	0.5	B	23.3,23.4	0.55,0.55	0.63
CH-34-K	0.5	"	9.1,8.8	0.21,0.20_5	0.28
CH-41-K	0.4	"	4.7	0.11	0.13
CH-42-K	0.5	"	20.1,19.6	0.47,0.46	0.52
CH-43-K	0.5	"	17.5	0.41	0.45
		C	38.5	0.43	
CH-44-K	0.5	"	7.3	0.08_5	0.13
CH-45-K	0.5	A	11.5	0.55	0.59
		B	22.8,23.3	0.54,0.55	

* The source of the sample is indicated thus: MGH, Massachusetts General Hospital; PBBH, Peter Bent Brigham Hospital; CH, Children's Hospital. The last letter indicates the type of extract, crude (C) or ketonic (K).

solution is then washed once with water, evaporated to dryness, and the residue made up to a volume of 4 cc. with 95 per cent ethanol. When a 0.2 cc. sample of the solution is converted to the Girard derivative and a portion polarographed by the standard procedure, determinations can be made covering the range of 1 to 20 mg. of hormone per liter of urine.

Estrone—Since estrone is a 17-ketosteroid, it is not surprising that the substance was found to give very much the same polaro-

graphic response as the androgens when condensed with the Girard reagent by the standard procedure. A discharge occurs at the same potential level, the form of the wave is similar, and the only noticeable difference is that the wave span is slightly less, whereas if the polarographic discharge followed the stoichiometric relationship the hormone of lower molecular weight would show the greater discharge. A few experiments were made with increasing amounts of estrone in the region of low concentration and the results, recorded graphically in Fig. 9, show that a direct propor-

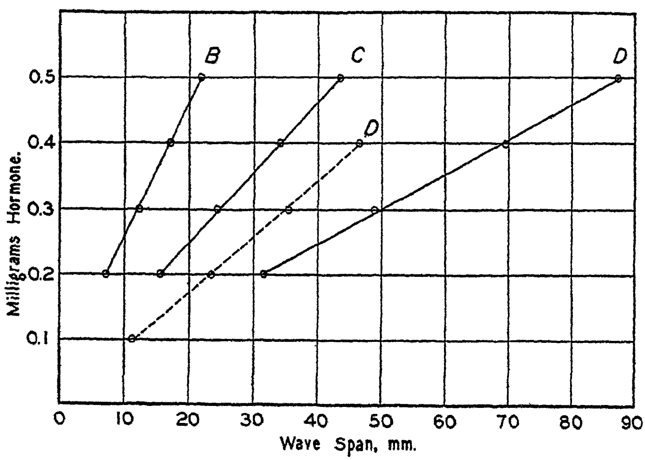


FIG. 9. Proportionality of wave span to weight of pure hormones at Sensitivities B, C, D. The solid lines represent estrone-Girard derivative, mg. content of total sample processed (4 times that polarographed). The dash line represents testosterone in alkaline isopropanol, mg. of material polarographed.

tionality exists between wave span and weight of pure hormone in the region covered. The determination of the hormone in urinary extracts would thus seem feasible.

Comparison with 3-Ketosteroids—Cholestanone and coprostanone, saturated 3-ketones having the two possible configurations at the 5 position, were condensed with Girard's reagent in the usual way and examined polarographically. Neither derivative gave any indication of a characteristic wave and there was no break in the diffusion current curve before the electrolytes began to show

terminal discharge. This unexpected observation was substantiated by an examination of the pure Girard derivative of cholestanone, which was isolated in a crystalline condition, for this showed the same behavior. The most obvious structural difference between a 3- and a 17-ketosteroid is that the carbonyl group is located in a six-membered ring in the former case and in a five-membered ring in the latter. For comparison, we therefore investigated cyclohexanone and cyclopentanone by the same procedure, with the results shown in Fig. 10. Cyclohexanone (Curve 1) although not completely indifferent gave no very distinct sign of a polarographic wave, whereas cyclopentanone (Curve 3) gave a

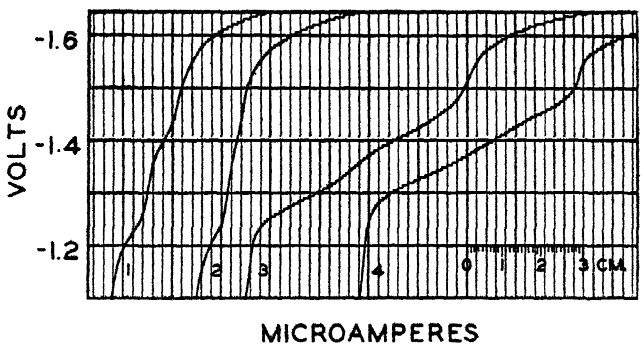
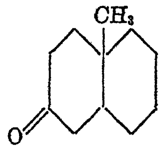
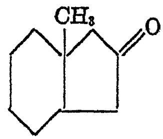


FIG. 10. Girard derivatives of cyclic ketones (0.5 mg. of each, standard procedure). Curve 1, cyclohexanone; Curve 2, *cis*-9-methyl-3-decalone; Curve 3, cyclopentanone; Curve 4, *cis*-8-methyl-2-hydrindanone.

wave of very extended span and of typical, if slightly irregular, form. As a further test case, comparison was made between synthetic model compounds kindly supplied by Professor R. P. Linstead; namely, *cis*-9-methyl-3-decalone, (I) (26), and *cis*-8-methyl-2-hydrindanone, (II) (26, 27). The six-membered ring ketone (I) (Curve 2) behaved very similarly to cyclohexanone,



(I)



(II)

while the five-membered ring compound (II) gave a wave corresponding to that of cyclopentanone. The difference in the response of the 3- and the 17-ketosteroids therefore is definitely attributable to the difference in the ring size. Considered with reference to the assay of steroids, the inert character of the 3-ketones lends further specificity to the polarographic method of determining the 17-ketonic hormones.

α,β-Unsaturated Ketones

Direct Determination in Alkaline Isopropanol—After trial had been made of various solvents and electrolytes, the following scheme was adopted for the direct polarographic characterization of α,β-unsaturated ketones of the sterol and hormone series. A

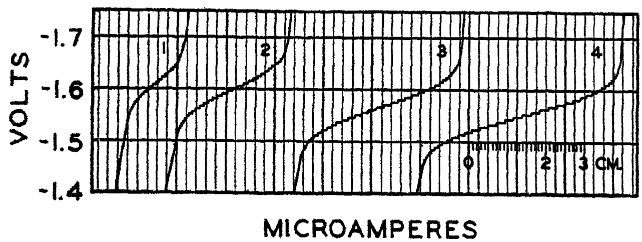


FIG. 11. Testosterone in alkaline isopropanol (fresh solutions, Sensitivity D). Curve 1, 0.1 mg. of hormone, wave span 11.2 mm.; Curve 2, 0.2 mg., 23.3 mm.; Curve 3, 0.3 mg., 35.3 mm.; Curve 4, 0.4 mg., 46.4 mm.

solution of the sample in 1 cc. of isopropanol was measured into the cell and treated with 0.5 cc. of aqueous 0.25 N tetraethylammonium hydroxide and 1 cc. of water. The solution was deaerated and polarized over the potential range from -1 to -2 volts and the span of the wave was measured in mm. by the method described above.

Typical results, obtained with increasing amounts of testosterone, are shown in Fig. 11. The curves are sharply defined, and over the limited range of concentration investigated the relationship between wave span and weight is strictly linear, as shown by the plot of the four results included in Fig. 9 (dotted line). This chart permits a direct comparison with the results for a typical Girard derivative, when allowance is made for the 4-fold difference

in the weights of the material polarographed. On analysis at Sensitivity D, 0.2 mg. of testosterone gives a wave span of only 23.5 mm., whereas 0.2 mg. of estrone in the form of the Girard derivative shows a span of 4 × 31.5 = 126 mm.

A point of interest in Fig. 11 is that the half wave potential recedes to less negative levels as the concentration of hormone is increased. A potential drift in the opposite direction was observed when the stability of testosterone in the alkaline solution was tested (Fig. 12). Relatively little difference is observable between the wave span of a very fresh solution (Curve 1) and that of the same solution after standing under nitrogen for over 2 hours (Curve 6). A parallel experiment with desoxycorticosterone ace-

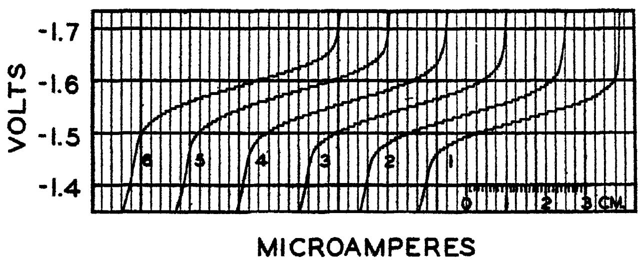


FIG. 12. Testosterone (0.4 mg.) in alkaline isopropanol polarographed at Sensitivity D after varying periods of time. Curve 1, 2 minutes after mixing; Curve 2, 12 minutes; Curve 3, 32 minutes; Curve 4, 52 minutes; Curve 5, 72 minutes; Curve 6, 132 minutes.

tate is recorded in Fig. 13. Here there was little if any change in either the half wave potential or the wave span until after about 1 hour, when evidence of decomposition became apparent (Curve 6).

All Δ^4-3-ketosteroids investigated exhibited much the same polarographic behavior in the alcoholic isopropanol solution, as shown in Fig. 14 which gives the curves obtained under comparable conditions with equal weights of a number of the substances of this series. The blank determination (Curve 1) shows that no discharge of electrolytes occurs in the potential range concerned. Corticosterone (Curve 2) gives a wave similar in form and potential to that of cholestenone (Curve 3), indicating that the additional carbonyl group at the 20 position in the side chain does not contribute to or influence the polarographic discharge. 17-Hydroxy-

dehydrocorticosterone (Curve 4) has two carbonyl groups in addition to the α,β-unsaturated carbonyl at position 3, but these at most exert a slight modifying influence, for the curve is in the same potential range and deviates from the characteristic form only in

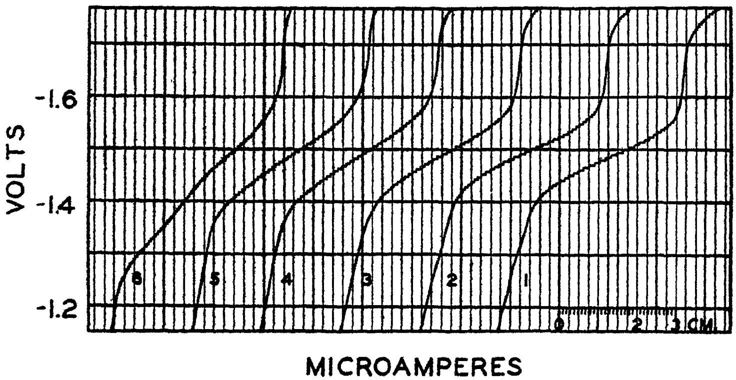


FIG. 13. Desoxycorticosterone acetate (0.4 mg.) in alkaline isopropanol polarographed (Sensitivity D) after the time intervals detailed in Fig. 12.

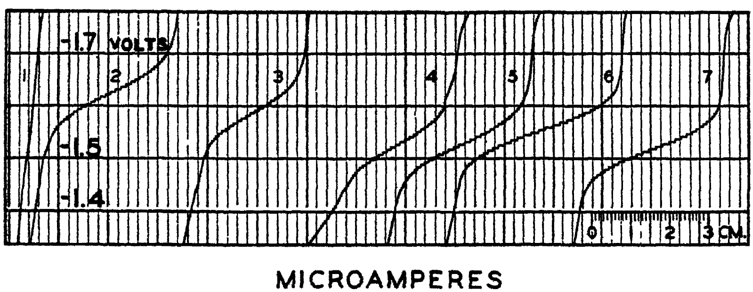
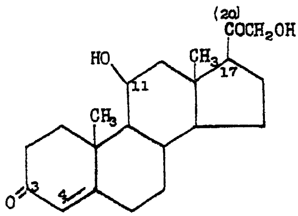


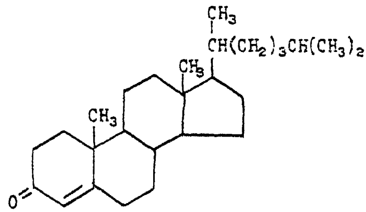
FIG. 14. Comparison of various α,β-unsaturated ketones (0.3 mg.) in alkaline isopropanol (Sensitivity D). Curve 1, blank; Curve 2, corticosterone; Curve 3, Δ^4-cholestenone; Curve 4, 17-hydroxydehydrocorticosterone; Curve 5, desoxycorticosterone acetate; Curve 6, testosterone; Curve 7, progesterone.

rather minor respects. Desoxycorticosterone (not shown) gives a tracing very similar to that of its acetate (Curve 5), and the curves for testosterone and progesterone (Curves 6 and 7) are of the same characteristic type.

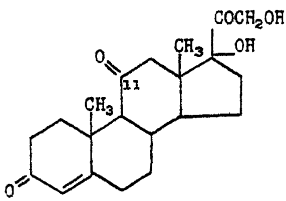
It is of interest to compare the extent of the discharge due to equal weights of the compounds (0.3 mg.) with reference to their molecular weights, as in Table III. The wave span tends to decrease with increasing molecular weight and with the first four compounds the relationship seems to be linear. The 17-hydroxy compound, however, is completely out of line with the other hormones and in the direction opposite to that which would signify a participation of the 11- and 20-carbonyl groups. Cholestenone likewise departs significantly from proportionality.



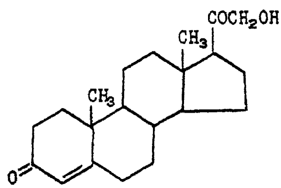
Corticosterone



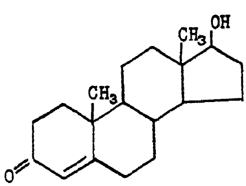
Δ^4-Cholestenone



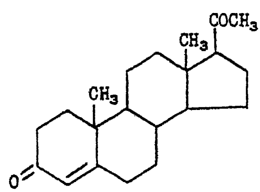
17-Hydroxydehydrocorticosterone



Desoxycorticosterone



Testosterone



Progesterone

The half wave potentials read directly from the charts are of purely empirical significance, since they are uncorrected and have not been evaluated in terms of a reference electrode. Furthermore, the potentials found above in an aqueous acetate buffer cannot be compared with those obtained in the alkaline isopro-

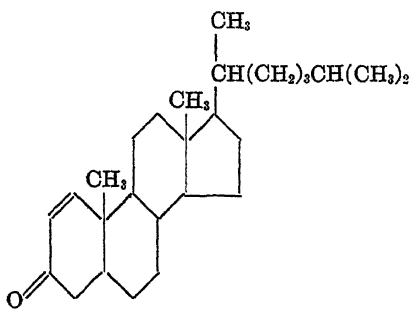
panol solution, for a reference basis is lacking. Nevertheless the uncorrected relative potentials observed in a given solvent under comparable conditions of concentration are of value in providing empirical indexes of structure. The above compounds are all Δ^4-3-ketones and give potentials ranging from -1.53 to -1.63 volts. We were fortunate in being able to investigate an α,β-unsatu-

TABLE III

Relation to Molecular Weights (Alkaline Analysis)

Compound	Mol. wt. (a)	Wave span (b)	$\frac{a \times b}{10{,}000}$
Testosterone	288.42	33.9	0.98
Progesterone	314.45	31.0	0.98
Corticosterone	346.45	29.7	1.03
Desoxycorticosterone acetate	372.49	26.7	1.00
17-Hydroxydehydrocorticosterone	360.44	12.2	0.43
Δ^4-Cholestenone	384.52	18.9	0.73

rated ketone of another structural type; namely, Δ^1-cholestenone. This substance was found to give a polarogram similar to that of Δ^4-cholestenone and of the same wave span. The potential in alkaline isopropanol was -1.43 volt, which is significantly below the level for the 3-ketones of the Δ^4 series. A potential in the range -1.5 to -1.6 volt is thus highly specific and distinguishes the Δ^4-unsaturated 3-sterones from saturated ketones of various types, and from at least one other α,β-unsaturated type. The list of natural hormones now shown capable of characterization by this method includes the important testosterone and progesterone,



Δ^1-Cholestenone

and three substances which have been isolated from the adrenal cortex and shown to have cortin activity.

Determination As Girard Derivatives—The polarographic behavior of the Girard derivatives of the α,β-unsaturated ketones was investigated both to see whether there are any advantages in the use of the derivatives in place of the free ketones and to determine the nature of the effect to be expected if unsaturated ketones are encountered in applying the above method for the analysis of urinary androgens. A given substance was condensed with excess Girard's reagent according to the standard procedure outlined

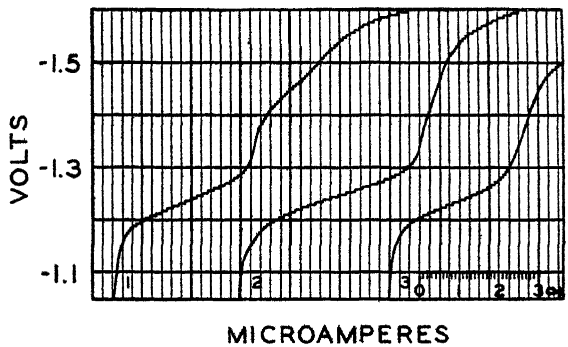


FIG. 15. Girard derivatives of α,β-unsaturated ketones (0.5 mg. sample, standard procedure, Sensitivity C). Curve 1, desoxycorticosterone acetate; Curve 2, testosterone; Curve 3, testosterone propionate.

above and one-fourth of the solution was prepared for polarographic analysis in the usual manner.

The behavior of unsaturated ketones having no other reducible groups is illustrated by the graph for testosterone, Curve 2 of Fig. 15, and also by Curve 3, for testosterone propionate. The waves are well defined and suited to accurate measurement, and the wave span is far greater than is obtained on polarographing a free ketone in alkaline isopropanol. Thus if the data of Figs. 14 and 15 for testosterone are placed on a common basis, it is seen that the Girard derivative gives a wave span 6.3 times as great as does the free hormone. This means that determination of the Girard derivative represents a far more sensitive method of analysis.

The half wave potential of the discharge attributable to the α,β-unsaturated ketonic group is approximately -1.23 volts, whereas that characteristic of the saturated 17-ketosteroid Girard compounds is about -1.44 volts. The difference is so pronounced that the potential of the wave serves as a reliable index of the type of ketone in hand. Furthermore, results obtained with compounds having two types of groups present in the same molecule indicate that these both leave an imprint on the polarographic chart. Thus desoxycorticosterone acetate (Fig. 15, Curve 1) gives a double wave; the first is quite distinct and occurs at a level (-1.23 volts) associated with the Δ^4-3-ketonic structure, while the

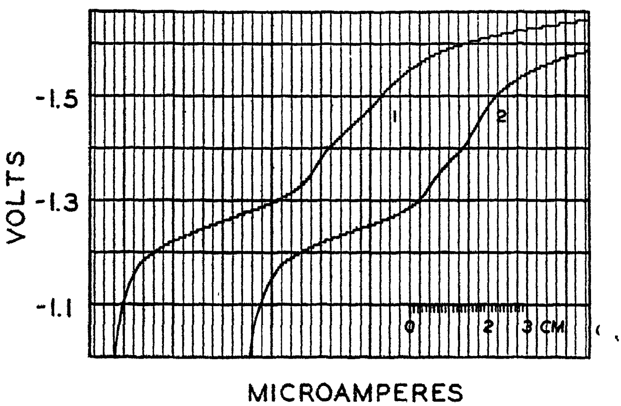
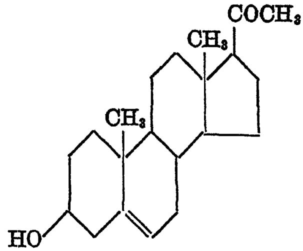


FIG. 16. Girard derivatives (0.3 mg. sample, Sensitivity C). Curve 1, corticosterone; Curve 2, 17-hydroxydehydrocorticosterone.

second wave is shorter and less clearly defined but is encountered at a considerably higher potential (about -1.45 volts). Curves similarly resolvable into two parts are given by corticosterone and 17-hydroxydehydrocorticosterone (Fig. 16), as well as by progesterone (Fig. 17, Curve 2). The fact that all of these compounds exhibiting an upper wave have a carbonyl group at the 20 position would suggest that this group is associated with the second discharge, and a secure basis for drawing this conclusion is provided by a comparison of the curves for progesterone and for Δ^5-pregnenol-3-one-20 (Fig. 17, Curve 1). The latter compound, in which the ketonic group at the 20 position constitutes the sole center in



Δ^5-Pregnenol-3-one-20

the molecule capable of condensing with the Girard reagent and forming a reducible group, gives a characteristic discharge at a

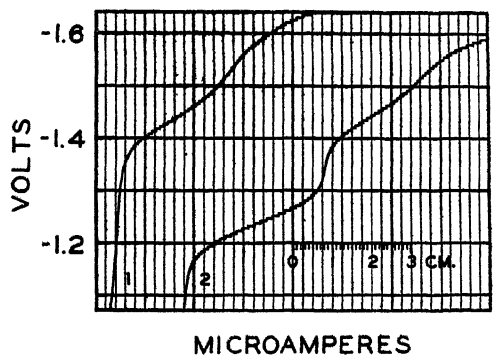


FIG. 17. Girard derivatives (0.5 mg. sample, Sensitivity C). Curve 1, Δ^5-pregnenol-3-one-20; Curve 2, progesterone.

half wave potential of about −1.45 volts, and it will be seen that this wave corresponds closely in position and form with the upper wave of the progesterone curve.

The marked difference in the potential of discharge of the Girard derivatives of unsaturated and saturated ketones suggested that the former might be determinable in the presence of the latter. An experiment to test this point is recorded in Fig. 18. A mixture of testosterone with double its weight of androsterone was condensed as usual with Girard's reagent; a sample when polarographed gave the double wave Curve 3. The lower wave clearly corresponds to that found with pure testosterone (Curve 2), while the upper one is recognizable as that of androsterone (Curve 1);

in the mixture both potentials seem slightly depressed. The ratio of the two wave spans agrees closely with that of the weights of hormones taken. It thus appears that the polarographic method developed provides for the simultaneous determination of α,β-unsaturated ketones and 17-ketosteroids in mixtures. Another case studied is that of a mixture of desoxycorticosterone acetate and androsterone (Fig. 19). The mixture gives a double wave (Curve 1) clearly revealing the presence of the cortical steroid derivative. With suitable standardization based on the lower part of the curve, the type of cortin principle represented should be

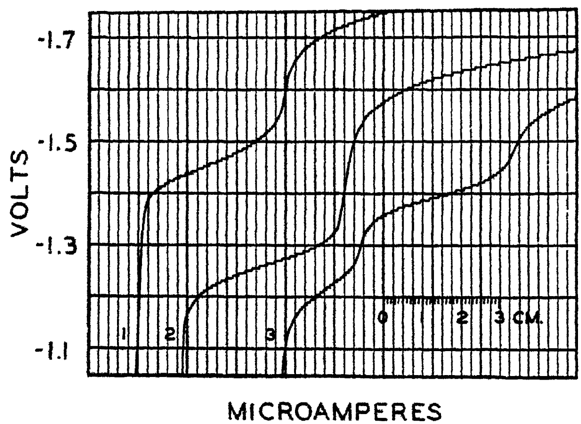


FIG. 18. Simultaneous determination of a saturated and an α,β-unsaturated ketone through the Girard derivatives. Curve 1, androsterone (1.0 mg.), Sensitivity B; Curve 2, testosterone (1.0 mg.), Sensitivity B; Curve 3, testosterone (0.2 mg.) and androsterone (0.4 mg.), Sensitivity C.

determinable in the presence of androgens normally occurring in urine. The upper portion of the curve for the mixture is doubtless a composite of the discharge due to androsterone and to the 20-ketonic group of the cortical steroid.

In Table IV the data for the wave spans recorded in the various charts just discussed have been reduced to the common basis of 0.5 mg. of total sample and Sensitivity C for comparison with the molecular weights of the uncondensed hormones. The deviation from a strict proportionality is well beyond the experimental error and there is less indication of a regularity here than in the series of determinations of the hormones in alkaline isopropanol solution.

The whole question of the stoichiometry of the polarographic analyses presents many perplexing problems, and the contrasting behavior of five- and six-membered ring ketones and of those having the carbonyl group at the 20 position raises further interesting problems which are difficult of theoretical interpretation. It

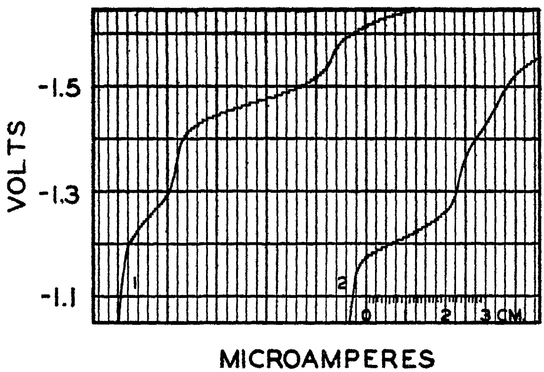


FIG. 19. Determination of a Δ^4-3, 20-diketone in the presence of a 17-ketosteroid (Girard derivatives). Curve 1, desoxycorticosterone acetate (0.2 mg.) and androsterone (0.4 mg.), Sensitivity B; Curve 2, desoxycorticosterone acetate (0.4 mg.), Sensitivity C.

TABLE IV

Relation to Molecular Weights (Girard Analysis)

Compound	Mol.wt. (a)	Wave span (b)	$\frac{a \times b}{10{,}000}$
Testosterone	288.42	45.0	1.30
Androsterone	290.44	35.5	1.03
Progesterone	314.45	35.0	1.10
Corticosterone	346.45	37.5	1.30
Testosterone propionate	346.49	45.0	1.56
Desoxycorticosterone acetate	372.49	31.5	1.17

is not clear why the Girard derivatives show a characteristic discharge covering a span several times greater than is observed with uncombined unsaturated ketones, unless it be that the result is caused by a summation of discharges due to the —C═N— linkage, the terminal ionic group, and possibly the amide carbonyl group. The theory of the polarographic phenomena, however, is of no

great moment to the objectives of the present work, and attention has been called to the idiosyncrasies encountered in order to indicate the limitations of the polarographic method and the precautions to be observed in its application to the quantitative determination of hormones. Standardization with reference to known compounds and in the presence of companion substances occurring in the extracts to be analyzed is all important. With adherence to this requirement, the method offers promise of fruitful application to a number of important problems involving the exact determination of ketonic androgens, estrogens, and cortical steroids.

SUMMARY

The 17-ketosteroids present in neutral urinary extracts can be determined accurately and rapidly by condensation with excess Girard's reagent and polarographic analysis of a suitably buffered aqueous solution of the reaction mixture. When similarly processed, saturated 3-ketosteroids are indifferent, ketonic estrogens are determinable, and steroids with a 20-ketonic group give a somewhat different response. The α,β-unsaturated Δ^4-3-ketosteroids are easily determinable by the same method and are readily distinguished from the 17-keto compounds, and even from Δ^1-3-ketosteroids, by a characteristically different potential of the polarographic wave. Hormones of the type of testosterone, progesterone, and corticosterone also can be characterized and determined by polarographic analysis in a mixture of isopropanol and aqueous tetraethylammonium hydroxide, although the determination of the same hormones in the form of the Girard derivatives offers advantages of specificity and sensitivity of analysis. By the latter method, α,β-unsaturated steroids can be determined in the presence of saturated ketonic androgens, in some cases with simultaneous determination of these androgens.

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STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

XI. THE MODE OF ACTION OF HEPARIN*

By MORRIS ZIFF AND ERWIN CHARGAFF

(*From the Departments of Biochemistry and Surgery, College of Physicians and Surgeons, Columbia University, New York*)

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Heparin requires the presence of a plasma protein to prevent the conversion of prothrombin to thrombin (1–3) and the coagulation of fibrinogen by thrombin (4). Quick (5) has shown that the factor which complements the action of heparin on thrombin is contained in the albumin fraction of serum prepared by Howe's method (6). This factor will be referred to in this paper as the *heparin complement.* In a preliminary communication (7) the authors have reported that crystalline albumin is inactive in complementing heparin. This has also been observed by Jaques and Mustard (8). In the present paper the distribution of the heparin complement in plasma and its interaction with thrombin are discussed.

EXPERIMENTAL

Methods—Thrombin was prepared by the method of Eagle (9) and *fibrinogen* in the usual manner by repeated precipitations with sodium chloride. *Heparin* was used in the form of the pure sodium salt.[1] All substances were dissolved in 0.85 per cent saline and adjusted to neutral pH.

The activity of plasma fractions in complementing heparin was determined as follows: 0.1 cc. of thrombin solution, 0.06 cc. of a heparin solution of appropriate concentration, and 0.06 cc. of the

* This work has been supported by a grant from the John and Mary R. Markle Foundation.

[1] We wish to thank Hoffmann-La Roche, Inc., Nutley, New Jersey, for the heparin preparation used.

protein fraction were mixed. After an incubation period, usually 3 minutes,[2] 0.2 cc. of fibrinogen solution was added. In control experiments 0.06 cc. of saline was substituted for either the heparin or the protein solution to be tested. The tubes were examined for clots at fixed intervals in order to avoid unnecessary agitation. Experiments were carried out at room temperature.

Relation between Thrombin and Heparin Complement—When decreasing quantities of albumin prepared according to Howe (6)

TABLE I

Effect of Decreasing Concentrations of Albumin Prepared by Howe's Method on Heparin Action

Experiment No.	N per cc. albumin solution	Clotting time of fibrinogen	
		10 sec.	15 hrs.
	mg.		
1	1.38	−	−
2	0.69	−	−
3	0.46	+	
4	0.23	+	

\+ = clot; − = no clot.

TABLE II

Effect of Decreasing Concentrations of Serum on Heparin Action

Experiment No.	Serum dilution	Clotting time of fibrinogen		
		30 sec.	1 min.	20 min.
1	Original	−	−	−
2	1:2	−	−	−
3	1:4	−	+	

\+ = clot; − = no clot.

in saline were mixed with fixed quantities of thrombin and heparin, and the mixtures added to fibrinogen, the coagulation of the latter was inhibited until a minimum concentration of the albumin was reached, at which the coagulation time fell sharply. These experiments are summarized in Table I. The thrombin, albumin, and fibrinogen were prepared from oxalated sheep plasma. The thrombin solution used was obtained by diluting five times the

[2] Longer periods produced no difference in results.

original solution resulting from the Eagle procedure. It clotted an equal volume of fibrinogen in 7 seconds. The heparin concentration was 0.1 per cent.

Similar results were obtained by adding normal human serum in increasing dilutions to the same clotting system in place of albumin, as shown in Table II.

The results shown in Tables I and II (in which for brevity numerous intermediate readings are omitted) demonstrate that the heparin complement acts only above a critical concentration.

Distribution of Heparin Complement in Plasma—A sample of citrated human plasma was half saturated with ammonium sulfate, and the globulins were separated. From the filtrate the fraction insoluble at 75 per cent saturation was removed. The filtrate thus obtained was concentrated to about half its volume by ultrafiltration through a collodion membrane at 600 mm. pressure (10), and brought to 100 per cent saturation. The resulting precipitate was removed. The fractions insoluble at 50 per cent and 75 per cent saturation were reprecipitated. The various plasma components were dialyzed in the cold against running water and physiological saline, and tested at neutral pH.[3]

Table III presents the activity of these fractions when tested with a thrombin solution diluted five times, and a fibrinogen solution, both prepared from human plasma. The heparin concentration was 0.3 per cent. A control solution containing thrombin, heparin, and saline clotted the required volume of fibrinogen in less than 15 seconds. The activity of a similar series of fractions obtained from normal horse serum on the same clotting system is given in Table IV.

In view of the activity displayed by the globulin fraction (see Experiment 1 in Tables III and IV) the amount of albumin contained in the human globulin (Table III) was determined by means of electrophoretic analysis. This was done in collaboration with Dr. D. H. Moore by the method of Tiselius (11) as modified by Svensson (12). The determination, carried out in 0.15 M sodium chloride and 0.02 M phosphate buffer at pH 7.40, indicated

[3] The filtrate, after removal of the fraction insoluble at 100 per cent saturation, was concentrated to 18 per cent of its original volume by ultrafiltration. This solution contained a minimal amount (0.28 per cent) of protein.

that the fraction contained 23 per cent albumin. The globulins were, therefore, reprecipitated by the method of Howe (6) and

TABLE III

Effect of Fractions from Human Plasma on Heparin Action

Experiment No.	Plasma fraction*	N per cc. protein solution	Clotting time of fibrinogen, min.			
			1	2	3	120
		mg.				
1	50%	2.0	−	−	−	−
2	50%	1.0	+			
3	75%	2.0	−	−	−	−
4	75%	1.0	−	−	−	−
5	75%	0.5	−	+		
6	100%	2.0	−	−	−	−
7	100%	1.0	−	−	−	−
8	100%	0.5	−	−	+	
9	Albumin	2.0	−	−	−	−
10	"	1.0	−	−	−	−
11	"	0.5	−	+		

+ = clot; − = no clot.

* 50, 75, and 100 per cent represent the plasma fractions insoluble at these ammonium sulfate concentrations. The albumin was prepared according to Howe (6).

TABLE IV

Effect of Fractions from Horse Serum on Heparin Action

Experiment No.	Plasma fraction*	N per cc. protein solution	Clotting time of fibrinogen			
			15 sec.	2 min.	20 min.	25 min.
		mg.				
1	50%	2.3	−	−	−	−
2	50%	1.2	−	+		
3	75%	1.1	−	−	−	+
4	75%	0.55	−	+		
5	100%	1.2	+			

+ = clot; − = no clot.

* 50, 75, and 100 per cent represent the plasma fractions insoluble at these ammonium sulfate concentrations.

tested again. It was found that the heparin complement was removed by this procedure, as shown by Table V.

Crystalline albumin from human plasma[4] displayed no heparin complement activity even when freshly prepared solutions of the

Table V

Effect of Globulin Fractions from Human Plasma on Heparin Action

Experiment No.	Globulin fraction No.*	N per cc. globulin	Clotting time of fibrinogen	
			1 min.	60 min.
		mg.		
1	G-1	1.20	−	−
2	G-2	1.28	+	

\+ = clot; − = no clot.

* Fraction G-1 was a globulin obtained from human plasma at 50 per cent ammonium sulfate saturation (see Experiment 1, Table III); Fraction G-2 the globulin resulting from the reprecipitation of Fraction G-1 by the Howe procedure (6).

Table VI

Crystalline Albumin and Heparin Complement Activity

Experiment No.	Albumin fraction*	N per cc. protein solution	Clotting time of fibrinogen						
			With heparin			Without heparin			
			30 sec.	25 min.	120 min.	30 sec.	3 min.	5 min.	10 min.
		mg.							
1	Crystalline	9.0	+			+			
2	"	4.5	+			+			
3	75%	2.7	−	−	−	−	−	−	+
4	100%	1.2	−	−	−	+			
5	Soluble at 100%	2.2	−	−	±	−	+		
6	Albumin (Howe)	1.4	−	−	−	−	−	−	+

\+ = clot; − = no clot.

* 75 and 100 per cent represent the fractions insoluble at these ammonium sulfate concentrations. The fraction soluble at 100 per cent (Experiment 5) was obtained by concentration of the filtrate after removal of the fraction insoluble at 100 per cent saturation.

solid were tested. In Table VI its activity is compared with that of a number of albumin fractions from human plasma in the pres-

[4] The human crystalline albumin was obtained from Dr. F. E. Kendall of the Research Division for Chronic Diseases, Welfare Island, New York. We are greatly indebted to Dr. Kendall for several specimens of this substance.

ence and absence of heparin (7). The fraction "soluble at 100 per cent" (Experiment 5) was obtained by concentration of the dialyzed filtrate after removal of the material precipitated at 100 per cent saturation. The clotting system was the same as in Table III.

Two samples of serum mucoid obtained from Dr. K. Meyer of this College were tested. One sample was from ox serum and contained N 11.4, hexosamine 6.3; the other was from horse serum, with N 11.9, hexosamine 5.2. Both substances were inactive.

The activity of albumin solutions stored in the cold did not decrease rapidly. Solutions prepared by Howe's method were still active after 50 days.

DISCUSSION

There exists a critical relationship between the anticoagulant activity of heparin and the relative concentration of heparin complement and thrombin (Tables I to III). For this reason the occurrence of clotting in a system containing thrombin, fibrinogen, and heparin may depend entirely on the concentration of the heparin complement. It is possible that the relationship between heparin complement and thrombin is a factor in pathological conditions. The concentration of this complement in the blood may be the determining factor in individual responses to heparin. It may be mentioned that recently a case of hemorrhagic diathesis has been reported (13) which was associated with a circulating anticoagulant, although no free heparin could be detected in the blood stream.

Crystalline albumin and serum mucoid are inactive. There was no appreciable difference in activity among the various other albumin fractions tested (see Tables III and IV) with the sole exception of Experiment 5 in Table IV. The globulin fraction prepared by ammonium sulfate precipitation contained sufficient albumin to be active; this was, however, removed by the Howe procedure. The occurrence of albumin in ammonium sulfate-precipitated globulin has been observed by McFarlane (14) using the ultracentrifuge. He found as high as 43 per cent albumin in a globulin precipitate from normal human serum. In our sample the presence of 23 per cent of albumin was detected by means of electrophoretic analysis.

It is at present impossible to state whether the ability to act as heparin complement is limited to a specific component of the albumin fraction. It may be significant that, with the sole exception of crystalline albumin, all albumin fractions tested displayed some activity. Speculation on the chemical reactions underlying the complement effect here discussed will have to be deferred until more data are available.

It is a pleasure to acknowledge the helpful advice rendered by Dr. H. S. Simms of this College and by Dr. F. E. Kendall of the Research Division for Chronic Diseases, Welfare Island, New York, in certain phases of the experimental work.

SUMMARY

The distribution of the protein factor in plasma which complements the effect of heparin on thrombin is discussed. Crystalline albumin from human plasma, in contrast to most other albumin fractions tested, is devoid of activity.

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THE ISOLATION OF 7(β)-HYDROXYCHOLESTEROL FROM THE SERUM OF PREGNANT MARES*

By O. WINTERSTEINER and JOHANA R. RITZMANN

(*From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, August 5, 1940)

Little information is available as to the chemical nature of that portion of the unsaponifiable matter of blood serum which is not cholesterol. The only other sterol known to occur is β-cholestanol, which Schoenheimer (1) isolated in small amounts from a pathological human serum. The presence of other compounds related to cholesterol has been repeatedly suggested, mainly on the ground that the unsaponifiable matter after removal of the cholesterol with digitonin contains substances giving the Liebermann-Burchard reaction (2–5). Another unidentified entity is the digitonin-precipitable "oxycholesterol" of Lifschütz (6, 2), demonstrable by the color reaction bearing his name, as well as by the trichloroacetic acid reaction of Rosenheim.

In the hope that the isolation and identification of some of these constituents of the serum would add to our yet very scanty knowledge of sterol metabolism, we subjected the unsaponifiable matter of pregnant mare serum to systematic fractionation. This type of serum was chosen primarily because the lipid fraction, a by-product in the manufacture of gonad-stimulating hormone, was available to us in large quantities. It may safely be assumed that at least qualitatively similar results would be obtained with normal horse serum. From the alcoholic fraction a digitonin-precipitable sterol, 7(β)-hydroxycholesterol, has been isolated. This compound is not new; Barr, Heilbron, Parry, and Spring (7) prepared it in 1936 by oxidation of cholesterol acid phthalate

* This work was carried out with the aid of a grant from the John and Mary R. Markle Foundation.

with alkaline permanganate, and proved its structure by conversion to 7-dehydrocholesterol, but to our knowledge it has not previously been encountered in biological material. Interestingly enough, its dextrorotatory epimer, 7(α)-hydroxycholesterol, has been shown by Haslewood (8) to occur in the unsaponifiable matter of ox liver. The identity of our isolated compound with the levorotatory isomer of Barr *et al.* was established by comparison with a sample prepared by the method of these workers. The melting point behavior of the natural as well as of the synthetic preparation showed some peculiarities, which we ascribe to the existence of solvated and solvent-free forms, which tend to give mixed crystals with each other. This obscured the mixed melting point determinations; however, crystal form, solubility properties, and specific rotations left no doubt as to the identity of the two preparations. Moreover, the properties of the respective dibenzoates showed full agreement.

The method of isolation of 7(β)-hydroxycholesterol is described in the experimental part. The sparing solubility of the diol in cold petroleum ether or pentane facilitated a partial removal of the cholesterol when the latter predominated in the mixture; for complete separation, adsorption of the acetates on aluminum oxide and fractional elution proved indispensable. The strong blue color given by the compound when dissolved in trichloroacetic acid (Rosenheim reaction) was employed as a guide in following the compound through the various fractionation steps.

Owing to the scattering of material unavoidable in an exploratory investigation and the undoubtedly heavy losses incurred in the fractionation, it is difficult to give an estimate of the amounts of 7(β)-hydroxycholesterol present in the unsaponifiable matter of the original serum. Starting from about 150 gm. of unsaponifiable matter we obtained from our best fraction 234 mg. of crude crystalline material, which yielded 84 mg. of the pure compound; an additional 70 mg. of somewhat less pure diol were obtained from the mother liquors and from other fractions. Taking into account the numerous side fractions, we may assume that the actual concentration in the unsaponifiable matter is probably several tenths of 1 per cent. On the other hand, we have evidence that not all of the chromogenic material in the final fractions is represented by 7(β)-hydroxycholesterol. In working up the

mother liquors of the diol we obtained a product crystallizable from absolute ether which exhibited a lower levorotation (−26°) than either 7(β)-hydroxycholesterol (−90°) or cholesterol (−38°). From methanol, in which it is much more soluble than 7(β)-hydroxycholesterol, this material deposited as a semicrystalline, gelatinous mass, reminiscent in appearance of the epimeric mixture of 7-hydroxycholesterols which is obtained by reduction of 7-ketocholesterol with aluminum isopropylate. The possibility exists that the dextrorotatory epimer, which is known to occur biologically (8), is likewise present in the diol fractions. The substance in question cannot be 7-dehydrocholesterol, because the latter is more strongly levorotatory than 7(β)-hydroxycholesterol.

An inquiry whether the isolated compound bears some relationship to the "oxycholesterol" which Lifschütz (6, 2) detected, by means of his color reaction, in the unsaponifiable matter of blood and of various organs, seems hardly profitable. Chemical and physical data, other than spectroscopic, on this entity are entirely lacking. Lifschütz was able to prepare from cholesterol by oxidation and other chemical means resinous products which possessed identical chromogenic properties and the composition $C_{27}H_{46}O_2$ required for a hydroxycholesterol (9), and the fact that such a preparation, made from cholesterol dibromide by treatment with sodium acetate, was shown by Rosenheim and Starling (10) to contain a diol later identified as Δ^4-cholestenediol-3,6 (11) has little bearing on the problem. The Lifschütz test is certainly not specific, as it is given not only by Rosenheim's diol but also by α- and β-7-hydroxycholesterol, and probably by all the numerous sterols that yield a blue color in the Rosenheim test.

Of greater interest are the numerous reports that "oxycholesterol" is formed from cholesterol by the influence of light, heat, and oxygen (12–16), and by aeration of aqueous colloidal solutions in the presence of soaps (15). Indeed, Bischoff (16) could not obtain a positive Lifschütz reaction on the unsaponifiable matter of blood, brain, and egg yolk when oxygen was excluded in the operations requiring heating, and he therefore doubted the existence of preformed "oxycholesterol." The question may then be asked whether the compound isolated by us is of true biological origin. Conditions, especially during the large scale operations, may have approximated those in the experiments quoted. To the

work done in our laboratory, beginning with the saponification of the lipid fraction, this applies only to a limited degree, since all procedures requiring heat or prolonged standing, as well as the vacuum distillations, were carried out in an atmosphere of nitrogen, and all solutions at rest were kept in the dark refrigerator. Nevertheless the mother liquors of the first crude cholesterol fraction gave a distinct Rosenheim reaction. A similar result, moreover, was obtained in two small scale experiments with 1.3 liters of pregnant mare serum and 3.7 liters of normal horse serum, which were collected from individual animals. In these all operations, from the drawing of the blood on, were conducted with the least possible delay and all practicable precautions against oxidation by air and light were taken. The succinates prepared from the cholesterol mother liquors gave the color reaction, which also points to the existence of preformed chromogenic products in serum. However, in working with the large quantities necessary in the search for new compounds it is virtually impossible to exclude all factors which might favor the formation of artificial "oxycholesterol," so the question of the source of any isolated compound giving the Lifschütz reaction is unanswerable. It is clear that the approach to this problem must be made by trying to establish the nature of the "oxycholesterol" formed from cholesterol. The yield of pure 7(β)-hydroxycholesterol represented a substantial portion (about 30 per cent) of the cholesterol-free, Rosenheim-positive fractions. On purely chemical grounds the possibility that 7(β)-hydroxycholesterol may have been formed by such accidental oxidation seems remote. If, on the other hand, *in vitro* oxidation to a well defined substance actually takes place with such facility, it can well be argued that an identical reaction may occur also under biological conditions; a line of thought, incidentally, which also underlies the work on the artificial preparation of "oxycholesterol" (Lifschütz, Rosenheim).

Our finding bears suggestively on the question of the biological origin of provitamin D_3, 7-dehydrocholesterol, which has been shown by Windaus (17) to occur in a variety of mammalian tissues. Both α- and β-hydroxycholesterol can be dehydrated by way of the dibenzoates to the provitamin, and may well also figure as intermediates in its biological formation.

EXPERIMENTAL

The starting material[1] was the lipid fraction (about 750 gm.) from some 150 liters of pregnant mare serum, obtained by precipitation of the serum proteins with acetone, which probably eliminated most of the phospholipids. The extract was saponified with 10 per cent potassium hydroxide in 90 per cent methanol by allowing the mixture to stand at room temperature for 7 days in an atmosphere of nitrogen. It was then exhaustively extracted with purified, low boiling petroleum ether in a continuous extractor. The material extracted by the first charge of petroleum ether was subjected to a second saponification for 12 days. The subsequent charges on concentration *in vacuo* deposited a clean looking, crystalline precipitate, which was separated by filtration. The complete removal of soaps from the rest of the petroleum ether-soluble products proved somewhat troublesome. Only after most of the soaps and other disturbing substances had been eliminated by extraction of the dry residue with acetone, in which they remained undissolved, could the remainder be effectively washed out of the ether or petroleum ether solutions with aqueous potassium carbonate. The crystallizable portion of the neutral fraction was then subjected to an extensive fractionation, in which methanol, petroleum ether, and acetone were employed as solvents. 50 gm. of almost pure cholesterol (m.p. 146–147°, $[\alpha]_D = -38.8°$ in chloroform), which gave no trace of blue color with trichloroacetic acid, were thus isolated. The rest of the material was largely contained in two fractions, 26.3 gm. of methanol-soluble products (Fraction A), and 70.8 gm. of a reddish brown oil, which was almost insoluble in methanol (Fraction B).[2]

[1] We wish to express our sincere thanks to Dr. E. Schwenk of the Schering Corporation, Bloomfield, New Jersey, for making this material available to us.

[2] The solubility properties of Fraction B suggested that it consisted at least in part of hydrocarbons. Small amounts of similar material were obtained from Fraction A after removal of the alcohols as acid succinates and of the ketones with Girard's reagent. These were subjected to further fractionation with methanol, and finally to fractional distillation in a high vacuum. No crystalline products could be isolated. Analysis of one of the distilled fractions showed that it consisted entirely of hydrocarbons. Since it appeared doubtful that all of the methanol-insoluble material was of biological origin, we prepared the unsaponifiable matter from 1.3

Fraction A was separated into alcoholic and non-alcoholic fractions by means of succinic anhydride and pyridine (18). The crude acid succinates were thoroughly extracted with petroleum ether at room temperature; the undissolved residue (10 gm.) consisted mainly of cholesterol acid succinate. The oily, petroleum ether-soluble material was dissolved in methanol and treated with an excess of lead acetate dissolved in the same solvent, in the hope that differences in the solubility of the resulting lead salts in organic solvents might effect a separation of the residual alcohols. A dark brown oil deposited, which was extracted with several portions of ether at room temperature, leaving a granular residue. The methanol supernatant was diluted with water and extracted with ether. Three lead salt fractions were thus obtained, (1) one soluble in methanol, (2) one soluble in methanol, but insoluble in ether, and (3) one insoluble in both these solvents. To remove the lead, each of these fractions was distributed between dilute hydrochloric acid and ether; the ether residues were saponified at room temperature (4 days) with 5 per cent methyl alcoholic potassium hydroxide. Not all of the saponified material could be extracted with petroleum ether after the addition of water. The aqueous phase was therefore concentrated *in vacuo*, and the extraction repeated with ethyl ether. The distribution of material in the resulting six fractions (petroleum ether residues, Fractions 1a, 2a, 3a, and ether residues, Fractions 1b, 2b, 3b)

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liters of pregnant mare serum and 3.7 liters of normal horse serum, and fractionated it in a similar manner. Strict precautions against accidental contamination with foreign matter were taken. The total lipid fractions were obtained by treatment of the serums with 5 volumes of ether-alcohol (1:1). The phospholipids were removed by precipitation with acetone; ethyl ether instead of petroleum ether was used for the extraction of the unsaponifiable matter. The yields of products free from alcohols and ketones indicated that no more than 5.6 and 3.7 per cent of the unsaponifiable material (from pregnancy serum and normal serum respectively) behaved like hydrocarbons. We conclude from these results that, though small amounts of hydrocarbons are probably present in serum, the corresponding fraction in our large batch must have been derived largely from contaminations introduced during the factory operations. The normal horse serum for the above experiments was made available to us by Dr. W. G. Malcolm of the Lederle Laboratories, Inc., Pearl River, New York, and the pregnant mare serum by Mr. W. O. Osborn of the Ben Venue Laboratories, Inc., of Cleveland, Ohio; we wish to extend to them our sincere thanks for these gifts.

was as follows: Fractions 1a 0.738 gm., Fraction 1b 0.247 gm., Fraction 2a 1.20 gm., Fraction 2b 0.364 gm., Fraction 3a 1.416 gm., and Fraction 3b 0.558 gm. All six fractions gave a strong Rosenheim reaction, but subsequent work showed that the largest amount of the diol was present in Fraction 3a. This fraction was extracted in a 50 cc. centrifuge tube, with 20 cc. of pentane, by stirring the suspension for a few minutes after short initial boiling. The insoluble material, a gelatinous mass, was centrifuged and the extraction repeated twice with the same amount of pentane, and then with two 10 cc. volumes of petroleum ether (b.p. 40–60°). The insoluble residue (433 mg.) was acetylated with acetic anhydride and pyridine at room temperature. The acetylated produce (514 mg.) was dissolved in 25 cc. of pentane and passed through a column of Brockmann's aluminum oxide (250 × 12 mm.). The column was washed in succession with 75 cc. of pentane, 100 cc. portions of pentane-benzene (9:1, 8:2, 6:4, 4:6), benzene, and finally acetone. The filtrate was cut for every ingoing 25 cc. The 8:2 pentane-benzene washings left on evaporation a colorless, mostly crystalline residue, which on purification yielded cholesterol acetate. The Rosenheim-positive products, colorless oils, were mostly contained in the 6:4 and 4:6 pentane-benzene washings (274 mg.). To assure the complete removal of cholesterol the absorption procedure was repeated on this fraction. The Rosenheim-positive material was distributed as before; the 6:4 and 4:6 pentane-benzene fractions were saponified separately with cold methyl alcoholic potassium hydroxide. Both solutions deposited needle-shaped crystals, which were centrifuged and washed with 90 per cent methanol (31 and 92 mg.). Since both preparations possessed the same melting point, namely 179–184°, no marked fractionation of the chromogenic material had apparently been effected. The remainder of the fraction was recovered by ether extraction of the mother liquors (111 mg.).

Fractions 1a, 1b, 2a, and the pentane-soluble part of Fraction 3a were all treated separately with digitonin in 80 per cent alcohol. An excess of the reagent and prolonged standing were necessary to make the precipitation complete. The sterols regenerated from the digitonides (209, 37.5, 550, and 288 mg. respectively) contained, as further fractionation and chromatographic analysis

showed, varying amounts of cholesterol and Rosenheim-positive products. Altogether about 200 mg. of the latter, free from cholesterol, were obtained from these fractions, but only 30 mg. of this amount could be recovered in form of the crystalline diol.

Undoubtedly the procedure of isolation as described could be simplified in several respects, with probable advantage to the yield. The fractionation of the succinates as lead salts, which resulted only in a scattering of the diol without effectively separating it from the cholesterol still present, seems unnecessary. The soluble succinates could preferably be saponified directly, and the remaining steps applied to the digitonin-precipitable products only. We plan to repeat the isolation from a new batch of starting material in this manner.

7(β)-Hydroxycholesterol—The combined crystalline fractions (123 mg.) yielded on two recrystallizations from methanol beautiful long needles melting at 183–186°. The whole preparation was dried for 3 hours at 110° and 12 mm. of Hg for the analysis and the determination of the specific rotation. The weight loss on drying was 7.3 per cent; calculated for 1 mole of methanol of crystallization, 7.37 per cent.

Analysis—$C_{27}H_{46}O_2$. Calculated. C 80.52, H 11.52
Found. " 80.36, " 11.25

$[\alpha]_D^{27} = -96.8°$ (0.995 % in chloroform)

A 3×10^{-3} M solution of the dried preparation in alcohol showed no specific absorption in the ultraviolet region above 220 mμ.

With trichloroacetic acid the compound gives a deep blue color. The green pigment formed with Lifschütz's reagent, prepared as described by Blix and Löwenhielm (15), exhibited the typical band at about 630 mμ.

The digitonide crystallizes from 80 per cent alcohol in beautiful small needles. Precipitation takes place more slowly than from cholesterol solutions of comparable strength.

The melting point of the original preparation (186°) was slightly depressed (183°) after drying at 110°. On recrystallization from methanol crystals indistinguishable in appearance from those of the undried sample were obtained, but their melting point was considerably lower (170–173°) and could not be raised by further crystallization. Still more puzzling was the behavior of the

material recovered from the chloroform solution used in the rotation experiment. It proved to be much more soluble in methanol than the original crystals; seeding with the sample melting at 183° was of no avail. Large rods melting at 154–157°, quite different in appearance from the long, fine needles of the higher melting samples, were finally obtained from a small volume of methanol on prolonged standing in the refrigerator. This preparation lost no weight on drying at 110° and 12 mm. of Hg for 1 hour. As was ascertained on another sample, prolonged heating at that temperature leads to decomposition. The same is true of the higher melting preparations.

Analysis—$C_{27}H_{46}O_2$.	Calculated.	C 80.52, H 11.52
	Found.	" 80.42, " 11.47

44.5 mg. of the diol, melting at 167–175°, were recovered from the combined mother liquors of the original crystals. The melting points on three subsequent recrystallizations from methanol were 175–181°, 175–182°, and 176–179°. The last crop was analyzed after drying *in vacuo* at room temperature.

Analysis—$C_{27}H_{46}O_2 \cdot CH_3OH$.	Calculated.	C 77.35, H 11.60
$C_{27}H_{46}O_2 \cdot H_2O$.	"	" 77.08, " 11.51
	Found.	" 77.30, " 11.04

The product was then recrystallized once more from methanol. The melting point was now 167–171°. The optical rotation was determined on the desiccator-dry material. $[\alpha]_D^{27} = -91.2°$ (0.704 per cent in chloroform).

Synthetic 7(β)-hydroxycholesterol, prepared by the method of Barr *et al.* (7), showed the same irregularities in melting point behavior. In fact we never succeeded in raising the melting point to that of the diol isolated from serum, namely 186°, which corresponds to the value given by the English workers (185°). The highest melting point attained was 179°, but on subsequent recrystallization it fell to 169°. As with some preparations of the natural compound, irregular rises and drops were observed in the intermediate crystallizations. On account of these difficulties the mixed melting point determination could be carried out only with products differing by 8° in their melting points (171° and 179°). The mixed sample melted at 176°. The preparation

lost 4.24 per cent of solvent on drying at 110° and 12 mm. of Hg. The weight became constant after 1 hour.

Analysis—$C_{27}H_{46}O_2$.	Calculated.	C 80.52, H 11.52
	Found.	" 80.60, " 11.62

The specific rotation ($[\alpha]_D^{27} = -89.9°$) of the (desiccator-dry) synthetic product, m.p. 169°, compared well with the value, −91.2°, obtained with a natural preparation melting at 171°. Furthermore, a weighed sample of the same material was dried to constant weight at 110° at 12 mm. pressure and its rotation determined. $[\alpha]_D^{32}$ on the basis of the original weight was again −89.9°, and −94.5° on the basis of the dry weight. It is probable that the value given by Barr *et al.*, $[\alpha]_D^{19} = -86.4°$, was obtained with a preparation containing solvent. When the synthetic sample recovered from the chloroform solution was recrystallized from methanol, it exhibited the same peculiarities as the natural material, and a highly concentrated solution finally yielded rods mixed with a small amount of needles.

The weight loss on drying in the above experiment was only 5.50 per cent; the natural compound, m.p. 186°, under identical conditions, lost 7.3 per cent, which would account for 1 mole of methanol of crystallization. Taking into account all the observed facts, we are inclined to believe that the diol exists in two modifications, a high melting form, m.p. 186°, crystallizing in needles which contain 1 mole of methanol, and a low melting form, m.p. 154–157°, crystallizing in rods without solvent of crystallization. The products with intermediate melting points and solvent content are obviously mixed crystals of the two forms. If once the low melting form, which seems to be the more stable one, is produced, for instance, by drying, or the use of chloroform as a solvent, it is apparently not possible to secure again the fully solvated form by crystallization from methanol.

7(β)-Hydroxycholesterol Dibenzoate—The dibenzoate was prepared from 40 mg. of a preparation of the natural diol melting at 173° by allowing its solution in 1 cc. of pure pyridine and 0.5 cc. of benzoyl chloride to stand at room temperature for 24 hours. The reaction mixture was worked up in the usual way. The crystals obtained from the ether residue were washed with cold methanol, and then recrystallized once from 1:1 methanol-acetone,

and twice from absolute alcohol. 33 mg. of fine needles, m.p. 155–156.5°, were obtained.

Analysis—$C_{41}H_{54}O_4$. Calculated. C 80.60, H 8.92
Found. " 80.41, " 9.15
$[\alpha]_D^{28} = -112.5°$ (0.68 % in chloroform)

A synthetic preparation (m. p. 169°) yielded on benzoylation needles of identical appearance and melting point (155–157°); this is somewhat higher than the melting point reported by Barr *et al.* for their dibenzoate (150–151°). The melting point of a sample mixed with the dibenzoate of the isolated compound showed no depression. $[\alpha]_D^{31} = -110.4°$ (0.79 per cent in chloroform); Barr *et al.* found $[\alpha]_D^{19} = -105.3°$.

Our attempts to prepare the crystalline diacetate (m.p. 122°) described by Barr *et al.* were unsuccessful. Both preparations, from the natural and from the synthetic diol, failed to crystallize from dilute alcohol as well as from pentane. Distillation in a high vacuum at 120–140° likewise yielded only an oily product.

SUMMARY

A sterol not hitherto encountered in biological material, 7(β)-hydroxycholesterol, has been isolated from the unsaponifiable matter of pregnant mare serum. Its identity was established by comparison with the synthetic compound which Barr, Heilbron, Parry, and Spring prepared by oxidation of cholesterol acid phthalate with permanganate.

The microanalyses reported in this paper were carried out by Mr. William Saschek.

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STUDIES ON PITUITARY LACTOGENIC HORMONE

IV. TYROSINE AND TRYPTOPHANE CONTENT*

By CHOH HAO LI, WILLIAM R. LYONS, AND HERBERT M. EVANS

(*From the Institute of Experimental Biology and the Department of Anatomy, University of California, Berkeley*)

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It has been shown (1) that the solubility of sheep lactogenic hormone is greater than that of beef lactogenic hormone in 0.01 N HCl solution. On the other hand, beef hormone was shown to be more soluble than sheep hormone in 0.1 M citrate buffer at pH 6.36. It is therefore likely that as compared with beef, sheep lactogenic hormone possesses more basic groups or fewer acidic groups in the molecule. Green *et al.* (2) have shown that horse and human carboxyhemoglobins differ in solubility, as demonstrated by the salting-out effect of phosphate ion, and the difference between these two proteins has also been shown by amino acid determinations (3, 4).

Of the amino acids, tyrosine and tryptophane are particularly interesting, the former usually being considered one of the constituents[1] essential for the specific biological rôle of certain proteins. Its importance for the antigenicity of proteins is generally recognized. Furthermore, the phenolic hydroxyl groups contribute, to some extent, to the dissociation constants of proteins. The tryptophane content (6) is especially useful in estimating the minimal molecular weight of proteins, for this amino acid usually constitutes but a small fraction of the molecule.

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[1] We (5) have recently shown that the tyrosine molecule is important for the biological activity of lactogenic hormone (iodination experiments).

EXPERIMENTAL

The lactogenic hormone prepared from both beef and sheep pituitary, essentially in the manner described previously (7) contained approximately 30 I.U. in 1 mg. and behaved as a single substance in electrophoresis (8, 9) and solubility experiments.

Lugg's modification (10) of Folin and Ciocalteu's method was used to determine the tyrosine and tryptophane content. 30 mg. of the final hormone preparation were dissolved with 0.5 cc. of 5 M NaOH in a small Pyrex test-tube. The sealed test-tube was then put into a steam bath for about 35 hours. The hydrolysate was then diluted with water to 10 cc. in a volumetric flask. 1 cc. of 5 N H_2SO_4 was added to 4 cc. of centrifuged solution and the determination of tyrosine and tryptophane was carried out essentially as described by Lugg. The color developed was measured in the Cenco-Sanford-Sheard photelometer with a blue filter. The tyrosine and tryptophane content of the solution was read off from a calibration curve which was made with pure tyrosine and tryptophane.

Results

Table I gives the tyrosine and tryptophane content of three preparations of sheep and beef lactogenic hormone respectively. Each value is an average of at least two determinations. It will be seen that the tyrosine content of beef hormone (5.73 per cent) is definitely higher than that of sheep (4.53 per cent), while the tryptophane content[2] is practically the same in both. It is not likely that the difference in the solubility behavior of these two proteins can be completely explained by their tyrosine content and further determinations of the other amino acids will be necessary to explain this phenomenon.

It is of interest to note that the tyrosine and tryptophane content of lactogenic hormone as given by Riddle and Bates (11) differs from our results. They reported that their preparations contain about 2.0 per cent tyrosine and 3.0 per cent tryptophane. Recently White and Lavin (12) demonstrated the presence of

[2] The experimental error of tryptophane determination is shown by Lugg to be at least 5 per cent. The difference existing between beef and sheep preparations is obviously within the experimental error.

TABLE I

Tyrosine and Tryptophane Content of Lactogenic Hormone As Prepared from Beef and from Sheep Pituitaries

Preparation No.	Origin	Tyrosine	Tryptophane	Preparation No.	Origin	Tyrosine	Tryptophane
		per cent	*per cent*			*per cent*	*per cent*
L283	Beef	5.64	1.37	L288	Sheep	4.63	1.30
L287	"	5.92	1.47	L292	"	4.20	1.28
L293	"	5.63	1.10	L299	"	4.75	1.00
Average......		5.73	1.31			4.53	1.19

tyrosine and tryptophane in their preparations by the ultraviolet absorption spectrum but no quantitative data were given.

As calculated from the tryptophane content, the minimal molecular weight of pituitary lactogenic hormone of both sheep and beef origin would approximate 15,000. As far as we have been able to learn, no protein contains more than 3 molecules of tryptophane. It follows that, unless lactogenic hormone is an exception, its molecular weight cannot exceed 45,000.

SUMMARY

The tyrosine and tryptophane content of sheep and beef pituitary lactogenic hormone has been determined and it was found that they contained practically the same amount of tryptophane (1.19 to 1.31 per cent), whereas the tyrosine content of beef hormone (5.73 per cent) was higher than that of sheep hormone (4.53 per cent). This finding may be said to support the evidence previously obtained from solubility studies that lactogenic hormone exhibits a species specificity.

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A METHOD FOR THE DETERMINATION OF THIAMINE AND CERTAIN OF ITS METABOLIC PRODUCTS IN URINE

By ALFRED S. SCHULTZ, LAWRENCE ATKIN, AND CHARLES N. FREY

(*From The Fleischmann Laboratories of Standard Brands Incorporated, New York*)

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The fermentation method for the determination of thiamine (1, 2) has been successfully applied to the analysis of numerous plant and animal tissues (3, 4). It has also been applied to metabolism studies (5, 6) in which the thiamine content of urine was determined. As noted at the time, it was recognized that the stimulation of fermentation caused by urine was due not entirely to thiamine itself but in part, at least, to metabolic breakdown products of thiamine. This partial lack of specificity (only partial since the non-thiamine stimulatory substances are considered to be related to thiamine) did not appear to affect the conclusions of those studies. It should be valuable, however, to be able to distinguish between thiamine and its breakdown products, because such information may have clinical significance.

As reported elsewhere (7), we have found that the oxidation of thiamine to thiochrome renders it inactive with regard to the fermentation test. On the other hand the partial destruction (by splitting or otherwise) of the thiamine molecule renders it unable to form the thiochrome structure and hence it retains its activity for the fermentation test. The conditions necessary for the differential oxidation of the thiamine-related substances in urine form the subject of this communication. With this method we have examined a series of human urines. By way of control the same samples were assayed by Light, Cracas, and Frey employing an improved rat growth method to be published shortly.

Apparatus

In addition to the usual equipment for making the fermentation test, a source of nitrogen (tank) and a manifold with three outlets are required. To each outlet is attached a short length of rubber tubing having a screw-clamp for constriction. Gas delivery tubes (about 6 mm. outside diameter) are directed to the bottom of cut down glass cylinders which are made by trimming broken 100 ml. cylinders to about the 30 ml. mark. The pressure of the nitrogen is conveniently maintained at about 2 cm. of Hg by a simple T-tube pressure release arrangement at some point between the tank and the manifold.

Reagents—

Potassium ferricyanide. 2 per cent aqueous solution, prepared daily.

Sodium hydroxide. 2 N.

Sulfuric acid. 1 N.

Isobutyl alcohol.

Thiamine. 10 γ per ml., prepared daily from a strong stock solution (1 mg. per ml.) which is kept refrigerated.

Boiled yeast solution. 3 gm. of moist yeast (such as is used to make the fermentation test) is suspended in water, acidified to Congo red, boiled, and made to 100 ml. when cool.

Procedure

In one cylinder (A) place about 20 ml. of the ferricyanide solution and pass a steady stream of nitrogen through it (200 to 300 bubbles per minute). In another cylinder (B) place about 20 ml. of the urine under test and pass N_2 through it also. In a third cylinder (C) place 5 ml. of NaOH and a drop of isobutyl alcohol. Pass N_2 through Cylinder C for 5 minutes and then by pipette transfer 4 ml. of urine from Cylinder B into Cylinder C, and follow this with 2 ml. of ferricyanide solution from Cylinder A. Now add 10 ml. of H_2SO_4 and stop the N_2 stream. Adjust the reaction to neutral (with neutral litmus paper) and, noting the volume, pour into one of the fermentation test reaction bottles. Rinse with a known volume of water. A subsequent fermentation test tells how much thiamine was destroyed by oxidation. Since experience shows that the efficiency of oxidation of thiamine in urine is less than 100 per cent, a parallel efficiency test with the

same urine is made. A mixture of the urine and the dilute thiamine solution is made so that 5 ml. of the mixture represent 4 ml. of urine and 1 ml. of thiamine solution; *i.e.*, 10 γ of thiamine. 5 ml. of this mixture are treated exactly like the original urine. The difference between the results with the original urine and the urine with added thiamine is used to compute the per cent of efficiency of the oxidation.

In the fermentation test proper, controls having two different known amounts of thiamine are employed. In this case it is desirable to use 0 and 2 γ as controls to avoid having to oxidize too much material. Furthermore it is best to have the mixture of salts, etc., present in the controls to avoid any interference due to a salt effect on the relation between the controls and the unknowns. Since it is desired to have present in the control reaction a mixture similar to that of the unknowns, a portion of the ferricyanide is reduced. 1 ml. of the boiled yeast solution is put through the same oxidation procedure as the urine. This quantity of yeast will have about 0.3 γ of thiamine before oxidation but afterwards will have only the slightest trace. Two of these are prepared, one each for the 0 and 2 γ controls.

Interpretation of Results

The urines are first analyzed by the usual fermentation test technique and the results expressed as fermentation vitamin B_1; *i.e.*, the stimulatory effect is measured in terms of thiamine. Table I gives the details of an analysis of two urines. Urine I is a normal 24 hour sample and Urine II represents the urine of a normal individual to whose diet 2.1 mg. of thiamine had been added.

The efficiency is obtained from the difference in the residual vitamin B_1 by fermentation, after oxidation of a portion of urine, with and without added thiamine. In the case of Urine I the difference is 1.3 γ, which means that 87 per cent of the added 10 γ has been oxidized.

Comparison of Results with Animal Tests

Table II gives the results of a series of analyses performed by both the method described and a rat growth method. When one considers that a precision greater than 15 per cent for vitamin B_1

is seldom claimed for animal methods, the results show a satisfactory agreement. The concentration of metabolic products

Table I

Thiamine and Metabolic Products of Thiamine in Urine by Fermentation Method

	Urine I	Urine II
24 hr. urine diluted to, *ml.*	2000	2000
Total fermentation vitamin B_1 (in thiamine equivalents), γ	700	2000
Fermentation vitamin B_1 in a 4 ml. aliquot, γ	1.4	4.0
Residue after oxidation of 4 ml. aliquot, γ	1.1	1.4
" " " " 4 " " + 10 γ thiamine, γ	2.4	2.9
Efficiency of oxidation (calculated from above), %	87	85
Total residue after oxidation (not corrected for efficiency), γ	550	700
Total residue after oxidation (corrected), γ	528	471
Total thiamine (true B_1), γ	172	1529

Table II

Total Daily Output by Fermentation and Rat Growth Methods

Subject No.	Urine composite	Diet	Total vitamin B_1 equivalents. Fermentation method	True vitamin B_1		Difference between methods
				Fermentation method	Rat growth method	
			γ	γ	γ	*per cent*
1	Sept. 7–10	Normal	806	498	498	0.0
2	" 7–10	"	848	342	400	+16.9
3	" 7–10	"	1020	630	750	+19.0
4	" 7–10	"	942	660	616	−6.7
5	" 7–10	"	853	427	475	+11.2
6	" 7–10	"	854	507	507	0.0
4	" 24–27	2100 γ thiamine per day	1856	1195	1352	+13.1
5	" 24–27	2100 " " " "	1748	1280	1173	−8.4
6	" 24–27	2100 " " " "	1907	1510	1359	−10.0

related to thiamine is obtained as the difference between total fermentation vitamin B_1 and the true vitamin B_1 and is most conveniently expressed in micrograms of thiamine equivalent.

SUMMARY

A method is described for the rapid determination of the true vitamin B_1 content of urine.

The content of thiamine breakdown products in the urine which are still active in the fermentation test is also determined by this method.

The method depends on the oxidative inactivation of the thiamine molecule by alkaline ferricyanide and the determination of vitamin B_1 by fermentation before and after oxidation.

The efficiency of the inactivation is determined by the proportion of a superimposed quantity of thiamine which is inactivated in a parallel test.

Comparison of the results with the results of rat growth tests shows a satisfactory agreement.

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THE EFFECT OF ADRENALECTOMY ON THE PHOSPHORYLATION OF VITAMINS B_1 AND B_2

By JOSEPH W. FERREBEE

(*From the Departments of Medicine and Neurology, College of Physicians and Surgeons, Columbia University, and the Neurological Institute, New York*)

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Verzár, Hübner, and Laszt (1) reported that livers of adrenalectomized rats contained less vitamin B_2 than livers of normal rats and that the vitamin was more easily removed by dialysis than was the case with normal animals. On the basis of this experiment they inferred that adrenalectomy interferes with the phosphorylation by which riboflavin is attached to the protein component of the yellow respiratory enzyme. Recently Laszt has apparently come to similar conclusions regarding vitamin B_1 (2). The following experiments were undertaken to obtain more information concerning the effect of adrenalectomy upon the phosphorylation of vitamins B_1 and B_2.

Methods

Young male white rats of the Wistar strain weighing 80 to 100 gm. were adrenalectomized bilaterally under ether anesthesia, by lumbar approach. Sham operations were performed on some of the litter mate controls. Five litters, twenty-one animals, were maintained following operation on a 10 per cent glucose solution containing approximately 10 γ of thiamine and 5 γ of riboflavin per cc. of solution. During the 1st postoperative week this solution also contained 1 per cent sodium chloride and 0.5 per cent sodium bicarbonate. Four litters, twenty-one animals, were placed on a low potassium diet and given sodium chloride in their drinking water during the 1st postoperative day.

Within a few days after salt treatment was stopped, symptoms

compatible with adrenal insufficiency developed in all adrenalectomized animals: cold extremities, weakness, anorexia, weight loss. Several animals died with these symptoms, others were killed by decapitation at various stages of insufficiency, and in conformity with the symptoms of adrenal insufficiency, the adrenalectomized animals were found to bleed much less than the controls. No adrenal tissue was found on gross examination at autopsy nor was any evidence found that death was due to causes other than adrenal insufficiency.

After the animals were killed, approximately 2 gm. samples of liver and kidney were weighed out as quickly as possible and ground to as near the same dispersion as possible in an ice-packed mortar. The tissue mash for dialysis was immediately suspended in 150 cc. of ice-cold distilled water and placed inside previously tested cellophane sacs suspended in cylinders containing 450 cc. of ice-cold distilled water. In a few experiments the tissues were dialyzed against 0.02 M phosphate buffer at pH 7.4 without essential difference in the results. A difference of pressure of 8 cm. of water was secured between the inner and outer fluids by creating a partial vacuum over the outside fluid. This difference in pressure kept the sacs well distended and made it easier to keep the tissue suspension well agitated by a continuous stream of washed air bubbles passed in from the compressed air line. The whole dialyzing system was immersed in ice water at 5°.

For determining the total amount of vitamin B_2 present before dialysis, duplicate samples of ground tissue were suspended in 0.03 M acetate buffer at pH 4.5, approximately 50 cc. per gm. of tissue, and the vitamins extracted by heating at 95° for $1\frac{1}{2}$ hours in the dark. The tissue suspensions in the cellophane sacs were similarly buffered and extracted following dialysis. The coagulated and extracted tissue was removed by filtration and 1 cc. of 10 per cent taka-diastase in 0.03 M acetate buffer was added to the filtrates which were then incubated overnight at 37°. A second extraction of the cooked tissues with hot acetate buffer for $1\frac{1}{2}$ hours yielded 5 to 10 per cent as much vitamin as that obtained in the first extraction. Duplicates of the first extraction differed by 5 to 10 per cent. The blank for the vitamin content of the taka-diastase solution was negligible.

The amount of vitamin removed by dialysis was determined by

subtracting the amount of vitamin found in the dialyzed sample from the amount of vitamin found in the undialyzed sample on the basis of micrograms of vitamin per gm. of fresh tissue. The amount of vitamin B_2 removed by dialysis was also determined by measurement of the B_2 fluorescence in the dialysate. This measurement agreed well with the measurement obtained by the difference between the vitamin contents of the dialyzed and undialyzed tissues.

Vitamin B_2 was estimated in the incubated extracts by a direct measurement of fluorescence similar to that described for urine vitamin B_2 determinations (3). On irradiation with blue light the incubated extracts gave an apparently pure greenish yellow riboflavin fluorescence quite different from the milky opalescence obtained when unincubated tissue extracts were irradiated. The standard values for riboflavin fluorescence in 0.03 M acetate buffer could be used in the calculations of vitamin B_2 concentrations, since very little correction was required for the slight difference between the optical density of the incubated extracts and the optical density of the pure buffer. Permanganate oxidation has never seemed to make the slightest difference in the vitamin B_2 fluorescence readings in these extracts and is now usually omitted as is also the test for B_2 fluorescence which involves hyposulfite reduction and reoxidation with atmospheric O_2. These procedures do not appear to contribute to the specificity of the vitamin B_2 determination under the circumstances of these experiments.

The amount of phosphorylated vitamin B_1 in the tissue extracts was estimated by determining the B_1 content of the tissue extracts before and after their incubation with taka-diastase. 1 gm. of potassium chloride was added to a 5 cc. aliquot of extract, and followed by 3 cc. of an alkali-ferricyanide mixture and 13 cc. of isobutanol. The thiochrome formed was immediately shaken out into the isobutanol and its fluorescence measured as described by Hennessey and Cerecedo (4). Blanks were run in some instances but can be omitted, particularly since they are close to the butanol blank. Before incubation almost all the vitamin B_1 of liver and kidney is phosphorylated if the tissues have been kept cold during the grinding and then quickly coagulated in the hot extracting buffer. Because phosphorylated vitamin B_1 (cocarboxylase) and its oxidation product, phosphorylated thiochrome, are only slightly

soluble in isobutanol, very little fluorescence appears in isobutanol extracts made from unincubated tissue filtrates. The increase in isobutanol-extractible fluorescence which follows taka-diastase incubation represents the amount of vitamin B_1 which was originally present in the tissue extract in a phosphorylated form.

Results

Effect of Adrenalectomy upon Concentration of Vitamins B_1 and B_2 in Rat Liver and Kidney—The data of Tables I to IV indicate that adrenalectomy did not affect the concentration of vitamins B_1

TABLE I

Effect of Adrenalectomy upon Vitamin B_2 in Rat Livers

The animals were maintained on a solution of glucose and vitamins B_1 and B_2.

Operation	Vitamin B_2 content per gm. fresh tissue	Per cent removed. 48 hr. dialysis
	γ	
None	27	60 ± 10
Unilateral adrenalectomy	24	60 ± 5
Bilateral "	25	50 ± 5
		4 hr. dialysis
Sham operation	15.5	15 ± 5
" "	16.3	18
Bilateral adrenalectomy	17.3	11 ± 3
" "	23	17
" "	26	18

and B_2 in the liver and kidney. No correlation could be found between the clinical condition of the animals and the concentration of vitamins in their tissues. An animal moribund with adrenal insufficiency might have as much vitamin in its liver and kidneys as a normal animal. Among the animals of Table I, which received vitamin in their drinking water, the concentration of vitamin B_2 in the livers of the adrenalectomized animals was actually greater than it was in the control animals. Considerable variation in vitamin B_2 concentration was found among the animals of Table IV but the variations were of the same order of magnitude in both the normal and the adrenalectomized group.

Effect of Adrenalectomy on Removal of Vitamin B_2 from Rat Livers by Dialysis—Two litters of animals maintained on a solution of glucose, salt, and vitamins B_1 and B_2 were used in the first part of this experiment (see "Methods"). In Fig. 1 the results of several

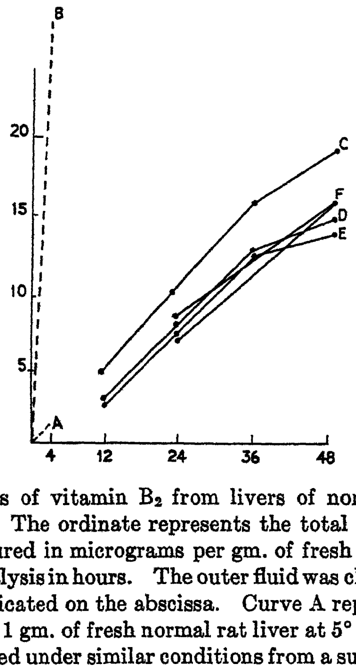

FIG. 1. Dialysis of vitamin B_2 from livers of normal and adrenalectomized animals. The ordinate represents the total vitamin B_2 removed by dialysis, measured in micrograms per gm. of fresh tissue; the abscissa, the duration of dialysis in hours. The outer fluid was changed at each of the time intervals indicated on the abscissa. Curve A represents the vitamin B_2 dialyzed out of 1 gm. of fresh normal rat liver at 5° in 4 hours; Curve B, the amount dialyzed under similar conditions from a suspension to which 50 γ of synthetic riboflavin had been added; Curve C, the progressive dialysis of riboflavin from 3 gm. of ground normal rat liver containing 81 γ of vitamin B_2; Curve D, a similar curve for dialysis of riboflavin from 2 gm. of liver taken from a unilaterally adrenalectomized animal; Curve E, a similar curve obtained with 1.8 gm. of liver from a completely adrenalectomized animal. The lines meeting at point *F* represent the dialysis of vitamin from fresh liver mashes of two adult rats, one normal and the other adrenalectomized bilaterally 9 days before the dialysis.

dialysis experiments are presented. Curve A indicates that the amount of vitamin B_2 which can be dialyzed out of 1 gm. of ground fresh normal rat liver at 5° in 4 hours is very slight, 2 γ or about 10 per cent of the total amount present. Curve B indicates that in the same length of time and under similar conditions of dialysis

28 γ of riboflavin can be dialyzed from a similar liver suspension to which have been added 50 γ of synthetic riboflavin. This represents a dialysis of approximately 50 per cent (28 − 2 or 26 γ out of 50 γ) of the riboflavin known to be present in this tissue suspension in a freely dialyzable form.

Curve C represents the progressive dialysis of riboflavin from 3 gm. of ground normal rat liver containing 81 γ of vitamin B_2. The points on the curve indicate the amount of riboflavin removed at the various time intervals of the experiment. Curve D is a similar curve for the dialysis of riboflavin from 2 gm. of liver taken from a unilaterally adrenalectomized animal. Curve E represents the dialysis of riboflavin from 1.8 gm. of liver taken from a completely adrenalectomized animal in good condition. The two lines meeting at point *F* represent the dialysis of vitamin from fresh liver mashes of two adult male rats that were maintained on a stock diet with added NaCl. One of these animals was normal; the other had been adrenalectomized bilaterally 9 days before the dialysis experiments were performed.

It is evident from Fig. 1 that adrenalectomy does not affect the shape of the dialysis curves. However, it is well to point out that this type of dialysis experiment, although suitable for comparative work, furnishes only presumptive information concerning the extent to which riboflavin is phosphorylated *in vivo*. There is no early break in Curves C to F to distinguish riboflavin which is free ante mortem from riboflavin which may be liberated by autolytic dephosphorylations, and since the slope of these curves is quite different from that of Curve B, it is entirely possible that part of the riboflavin in the 48 hour dialysates may have come from the slow postmortem breakdown of macromolecular compounds containing this vitamin.

In Table I are summarized the results of these experiments and others in which dialysis was continued for only 4 hours to minimize the effect of autolysis. The amount of riboflavin found in the dialysate after 4 hours was between 14 and 20 per cent of the total, independently of whether the animals had a sham operation or were completely adrenalectomized, and independently of whether they were in good condition or weak with adrenal insufficiency.

Effect of Adrenalectomy upon Phosphorylation of Vitamin B_1 in Rat Liver and Kidney—The experiments summarized in Tables II

and III demonstrate that adrenalectomy has no significant effect upon the percentage of the total vitamin B_1 which is found unphos-

TABLE II

Effect of Adrenalectomy upon Vitamin B_1 in Rat Livers

Diet	Operation	Vitamin B_1 content per gm. fresh tissue	Per cent removed by 48 hr. dialysis
		γ	
Low potassium	Sham operation	3.7	45
	" "	3.6	45
	" "	2.9	28
	Bilateral adrenalectomy	4.8	48
	" "	4.7	53
	" "	4.1	27
			Per cent not phosphorylated (taka-diastase method)
Low potassium, with vitamin B_1 (5–10 γ per cc.) in drinking water	None	12	14
	"	18	9
	"	22	10
	"	51*	25
	Unilateral adrenalectomy	18	7
	Bilateral "	13	15
	" "	12	11
	" "	10	14
	" "	56*	19
			Per cent not phosphorylated
Solution of glucose, and vitamins B_1 and B_2	None	8	15
	"	8	7.5
	"	8	7.5
	Unilateral adrenalectomy	11	27†
	Bilateral "	14	12
	" "	14	10
	" "	7	20
	" "	14	11
	" "	5	10
	" "	15	5

* Animal killed 40 minutes after a subcutaneous injection of 1 mg. of vitamin B_1.

† Tissue allowed to stand several hours in the ice box.

TABLE III

Effect of Adrenalectomy upon Vitamin B_1 in Rat Kidneys

The animals were maintained on a solution of glucose and vitamins B_1 and B_2.

Operation	Vitamin B_1 content per gm. fresh tissue	Per cent not phosphorylated
	γ	
None	5	24
"	9	8
"	8	18
Bilateral adrenalectomy	5	24
" "	8	9
" "	9	8
" "	6	40*

* Animal found dead.

TABLE IV

Effect of Adrenalectomy upon Concentration of Vitamin B_2 in Rat Liver and Kidney

The animals were maintained on a low potassium diet without added vitamins but with added salt during the first 3 postoperative days.

Operation	Liver		Kidney	
	Days post-operative	Concentration of vitamin B_2 per gm. fresh tissue	Days post-operative	Concentration of vitamin B_2 per gm. fresh tissue
		γ		γ
Sham operation	7	43	7	36
" "	7	40	10	23
" "	10	19	10	30
" "	10	20	10	30
" "	10	18	10	20*
Bilateral adrenalectomy	7	33	7	40
" "	10	25	7	32
" "	10	16	10	17
" "			10	15
" "			10	21*
" "			10	22*

* Animals maintained on a stock diet.

phorylated in extracts of rat liver and kidney. A comparison of the data of Table II indicates that the adrenalectomized rat is

well able to phosphorylate and store vitamin B_1 added to its basal ration. In view of what has been said about the difficulty of interpreting dialysis experiments it is significant that the percentage of vitamin B_1 removed by 48 hours dialysis was considerably greater than the percentage found unphosphorylated in fresh tissue extracts.

DISCUSSION

In a series of publications Verzár and his coworkers have presented experimental evidence which they interpreted as indicating that the cortex of the adrenal gland has some specific control over the phosphorylations involved in the utilization of glucose, fat, vitamin B_1, and vitamin B_2. They demonstrated impairment of fat and carbohydrate absorption in adrenalectomized animals and interpreted this finding as being related to a failure of phosphorylation in the intestinal mucosa (5, 6). They were able to maintain adrenalectomized rats with phosphorylated vitamin B_2 but not with the unphosphorylated vitamin and inferred from this observation that a corticoadrenal hormone controls the phosphorylation of riboflavin which makes possible the formation of yellow respiratory ferment (7). In substantiation of this view they presented evidence that the concentrations of vitamin B_1 and vitamin B_2 are less in the livers of adrenalectomized rats than in the livers of normal rats and that the ratio of phosphorylated to unphosphorylated vitamin is considerably decreased following adrenalectomy (1, 2).

Unfortunately experimental confirmation of this attractive hypothesis relating corticoadrenal function with phosphorylation (8) does not appear to be forthcoming. Deuel, Althausen, Barnes, and their coworkers have suggested that the impairment of fat and carbohydrate absorption observed by Verzár was due to shock and dehydration rather than specific lack of cortical hormone. In their experience (9–11) the absorption of fat and carbohydrate was normal when the adrenalectomized animals were given enough salt to prevent circulatory collapse. Moreover, Nelson (12) has found that phosphorylated riboflavin does not increase the survival time of completely adrenalectomized animals[1] and the experiments

[1] It is significant that both Verzár and Pijoan (13) found cortical tissue in the animals whose survival time they reported as increased by the administration of phosphoflavin (Pijoan, M., personal communication).

of the present paper indicate that the phosphorylation of both vitamin B_1 and vitamin B_2 is essentially normal following adrenalectomy.

SUMMARY

1. The phosphorylation of vitamins B_1 and B_2 is essentially normal in adrenalectomized rats.
2. The arguments against Verzár's thesis that adrenalectomy interferes with phosphorylation are briefly discussed.

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NICOTINIC ACID DERIVATIVES IN HUMAN URINE AND THEIR DETERMINATION*

By WILLIAM A. PERLZWEIG, EDWARD D. LEVY, and HERBERT P. SARETT

(*From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina*)

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This communication deals with a method of determination of nicotinic acid and its derivatives in human urine only, and with the application of the method to the study of the excretion of these substances in the urine normally and after the ingestion of nicotinic acid by human subjects.

We were led to the elaboration of the proposed method because we failed to obtain reproducible and concordant results with the methods (1–9) published since the discovery of the vitamin activity of nicotinic acid in 1937 (10). The difficulties we encountered may be grouped as follows:

The varying conditions of preliminary hydrolysis with acid did not insure complete hydrolysis of the known nicotinic acid derivatives to nicotinic acid, and with alkaline hydrolysis yielded much higher and variable values.

The other methods failed to take into account the presence in the urine of substances interfering with the final color reaction and of other extraneous pyridine derivatives, especially in smokers' urines.

* Part of the data of this article is taken from a thesis presented by Edward D. Levy in partial fulfilment of the requirements for the degree of Master of Arts in the Graduate School of Arts and Sciences of Duke University, June, 1940.

A preliminary report was made by the authors before the meeting of the American Society of Biological Chemists at New Orleans (*Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **133**, p. lxxiv (1940)).

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Decolorization of urine without loss of nicotinic acid presented a difficult problem.

In those methods in which decolorization was not employed, and the nicotinic acid content was estimated by extrapolation of values obtained from added known amounts of nicotinic acid, the extraneous amount of color in the original urine was so great that small errors in its determination led to very large errors in the relatively low calculated or extrapolated values.

The problem of the correction by any appreciable blank value due to the urinary pigments or of those arising during the manipulations is particularly formidable. No true blank value could be found by the omission of one or the other of the two color-producing reagents, since the pigments themselves apparently react to modify the nicotinic acid color.

Method

Reagents—

Concentrated acids, HCl, HNO_3, H_3PO_4.

12 N KOH solution.

Lloyd's reagent.

0.5 N KOH solution.

0.2 N H_2SO_4 solution.

$Pb(NO_3)_2$, powdered.

K_3PO_4, powdered.

CNBr reagent, saturated bromine water (3.4 per cent at 20°) prepared by shaking excess of bromine in a bottle for 2 hours, and kept in the refrigerator. Titrate a portion in ice water with freshly prepared 10 per cent NaCN of highest purity[1] to complete disappearance of the yellow color, avoiding an excess.

p-Methylaminophenol sulfate, a freshly prepared, saturated (about 5 per cent) solution of Eastman Kodak Elon in water.

Determination of "Total" Nicotinic Acid (Exclusive of Trigonelline)

Either freshly voided or toluene-preserved urine kept in the refrigerator is used. 20 ml. of urine with a specific gravity of about 1.020 are just neutralized (pH 8.0 to 8.5) to phenolphthalein with KOH solution, and evaporated to a thick syrup on a steam bath. This is transferred with 4 ml. of water and 6 ml.

[1] KCN is apt to give difficulties because of impurities.

of concentrated HCl to a 100 ml. Kjeldahl flask; 0.25 ml. of con centrated HNO_3 is added, also a few quartz chips, and boiled gently over a small flame of a microburner for 60 minutes, with a "cold finger" or Hopkins condenser. Care must be taken in the beginning until the foaming stops. Charring and darkening occur at first, but after 10 to 15 minutes the solution becomes light yellow. After cooling, the solution is made up to 20 ml. with water in a narrow 25 ml. graduated glass-stoppered cylinder.

15 ml. of this are transferred to a beaker, partially neutralized with 2 ml. of 12 N KOH, cooled, and adjusted to pH 1 with the glass electrode (or external indicator—methyl violet). 2 gm. of Lloyd's reagent are placed in a 22 × 175 mm. Pyrex test-tube previously graduated to contain 15 ml. of liquid in addition to the 2 gm. of Lloyd's reagent. The acid solution is transferred to this tube, being washed in with 0.2 N H_2SO_4 from a wash bottle. The tube is now closed with a rubber stopper and shaken rather vigorously a few times, the gas pressure released, and the tube is then shaken for at least 1 minute by inversion. The stopper and sides of the tube are washed down with 0.2 N H_2SO_4, the tube centrifuged briefly, and the clear supernatant fluid is decanted and discarded. The Lloyd's reagent sediment is washed twice by shaking with about 5 to 10 ml. of 0.2 N H_2SO_4 and centrifugation. After the last washing is decanted, 8 ml. of 0.5 N KOH are added and shaken for 1 minute with the Lloyd's reagent for elution, water is added to the 15 ml. mark, and the contents are mixed and centrifuged. The colored supernatant fluid is decanted into an ordinary Pyrex test-tube, 0.6 gm. of powdered $Pb(NO_3)_2$ is added, and the contents shaken; if the supernatant solution is too highly colored, 2 drops of concentrated NH_4OH are added, and the tube is shaken and centrifuged. The supernatant fluid is now of a light yellow or amber color. The excess of lead is removed by the addition of a pinch (0.2 gm.) of K_3PO_4 to alkalinity and centrifuging off the precipitate after 5 to 10 minutes.

Color Development—This is an adaptation of Bandier and Hald's method (4, 5). Two aliquots of 5 ml., representing 5 ml. of the original urine, are measured into small beakers, adjusted with 5 N H_3PO_4 to pH 4.5.[2] The solutions are transferred with a few

[2] We use a glass electrode, but an external indicator may be used with a standard buffer control for comparison.

ml. of water to Evelyn colorimeter tubes graduated at 20 ml. A standard solution containing 10 γ of nicotinic acid adjusted to pH 4.5 with H_3PO_4 is also set up in another tube, as well as a tube containing 100 mg. of KH_2PO_4 in water as a reagent blank. Of each pair of the tubes containing urine extracts one is kept for a control, while the other tube and those containing the standard and reagent blank are treated as follows: The tubes are immersed in a water bath at 75–80° for 5 minutes. 1 ml. of the freshly prepared CNBr solution is added to each of the tubes and they are kept in the bath 5 minutes longer. The tubes are cooled by immersion in ice water. 10 ml. of the Elon solution are added, the solutions diluted to the 20 ml. mark, mixed by inversion, and the tubes are placed in a dark cupboard for 1 hour, when they are read in the usual way in the Evelyn photoelectric colorimeter with the No. 400 filter; the galvanometer was set at 100 with the reagent blank tube.

The controls are merely diluted to the 20 ml. mark with water including 0.18 ml. of 2 N H_2SO_4 and are read with the same filter, the galvanometer being set at 100 with a tube containing water. The L values of the controls as given on the chart accompanying the colorimeter are subtracted from the values of the corresponding solutions in which the color was developed. The calculations are made from the value obtained with the standard solution.

Determination of Trigonelline

This procedure is based on our previous observation (11) that trigonelline, heated with strong alkali in the presence of ammonia (urea), yields a substance which gives a color identical with that of nicotinic acid and amounting to 70 per cent of the theoretical conversion under optimal conditions.

Test-tubes each containing 300 mg. of urea and 1.3 ml. of 12 N KOH solution and covered with small funnels are immersed for 5 minutes in a water bath in a beaker maintained at 75–80° on an electric hot-plate. Then 1.00 ml. aliquots of the HCl-HNO_3 hydrolysates prepared above are pipetted into the tubes, and the contents are mixed. The tubes are covered and left in the 75–80° bath for 45 minutes. The contents are washed into small beakers, with about 8 to 10 ml. of 0.2 N H_2SO_4. The solutions are brought to pH 1 (glass electrode) with about 1.5 to

2.0 ml. of concentrated HCl, and the further adsorption on and elution from Lloyd's reagent and all subsequent manipulations are exactly as given above for the acid-hydrolyzable derivatives. When 5 ml. aliquots are used for the color analysis they represent 0.33 ml. of the original urine, and the blank values are so small that they can be omitted and neglected. The values obtained in this color analysis include the above nicotinic acid derivatives determined after strong acid hydrolysis plus 70 per cent of nicotinic acid color yielded by the trigonelline in the alkaline hydrolysis. After the acid-hydrolyzable nicotinic acid is subtracted, the remainder is multiplied by the factor 100/70 to give trigonelline as nicotinic acid. This latter value, if desired, is converted into terms of trigonelline itself by the use of the factor 1.11 which represents the ratio of gm. molar equivalents of the two substances, 137:123.

Discussion of Methods

Acid Hydrolysis—From Ackermann's data (12) obtained on feeding nicotinic acid to dogs it was reasonable to suspect that human urine might, in addition to nicotinic acid, also contain the glycine conjugate (nicotinuric acid) and trigonelline; obviously the presence of free nicotinamide or coenzyme might also be expected. As far as it is known, of these four substances only trigonelline was actually isolated and identified by Linneweh and Reinwein (13) in normal human urine. These authors isolated 0.42 gm. of trigonelline from 40 liters of normal human urine from subjects using coffee, tea, etc., and 0.014 gm. from 11 liters of the urine of subjects who abstained from these substances.

We first studied, therefore, the conditions required for the complete hydrolysis of nicotinamide and of nicotinuric acid. In the case of the amide it was found that 1 hour's heating in a boiling water bath in the presence of 1 N HCl or 1 N KOH resulted in complete hydrolysis as indicated by the yield of the theoretical amount of nicotinic acid measured by any of the colorimetric procedures employed. In the case of the nicotinuric acid the behavior in this respect was quite different. Although relatively mild treatment with alkali, heating for 1 hour with 1 N KOH at 100°, gave complete hydrolysis, nicotinuric acid is

remarkably resistant to treatment with strong acid. It required quite vigorous boiling with 5 N HCl for 1 hour to obtain complete hydrolysis. In this respect the behavior of nicotinuric acid is very similar to that of hippuric acid (14).

Since there are reasons to believe that a considerable portion of the nicotinic acid is excreted in the form of nicotinuric acid, as will be shown below, it becomes obvious that one must employ a hydrolysis procedure either with at least 1 N alkali at 100° or with very strong acid at a higher temperature. The objection to the use of alkali here lies in our previously described observation (11) that when trigonelline is heated in an alkaline solution in the presence of ammonia it is transformed into a substance behaving like nicotinic acid, the yield of the latter depending upon the conditions.

We have found by extensive study of the conditions (concentration of reactants, temperature, and time) that heating at 75–80° for 45 minutes in 6 N KOH in the presence of urea gives an optimum conversion of trigonelline, amounting to 65 to 75 per cent of the nicotinic acid equivalent. In those methods for nicotinic acid in which alkaline hydrolysis is recommended variable amounts of trigonelline will be converted in this manner, and the nicotinic acid values will be erratically raised.

We, therefore, must depend upon the strong acid hydrolysis for the determination of what we designate as "total" nicotinic acid, including nicotinamide and nicotinuric acid, but exclusive of trigonelline.

For the determination of trigonelline in urine the initial acid hydrolysis is necessary to destroy interfering substances. Direct hydrolysis with 6 N KOH always yielded lower initial values and has given poor recoveries of added trigonelline unless subjected to the preliminary acid hydrolysis.

The data in Table I illustrate this point.

Decolorization and Removal of Interfering Substances—It was soon discovered that the problems of reproducibility of results, of proportionality of values when varying amounts of urine were taken for analysis, and of the recovery of added nicotinic acid (of the amide, nicotinuric acid, or of trigonelline) depended in the final analysis upon the successful removal of the colored substances and pigments either initially present in the urine or those which arise during the heating with acid and alkali.

The expedient of decolorization with charcoal first proposed by Vilter *et al.* (2) and by Swaminathan (7) did not yield in our hands satisfactory results. We have not been able to discover a single kind or brand of charcoal which did not remove variable amounts of nicotinic acid from pure aqueous solutions at pH values varying from 1 to 10 and at varying temperatures from 20–100°. Neither did the recently proposed method of Melnick and Field (9) in which charcoal is used in a medium containing 40 per cent alcohol and 2 N HCl permit us to recover the initially present nicotinic acid in pure solutions or in urine. Other adsorbing media were tried, including commercial varieties of bentonite, franklinite, kaolin, alumina, etc., with no better success. In fact, it was found that nicotinic acid is adsorbed very readily

TABLE I

Need for Preliminary Acid Hydrolysis in Trigonelline Determination in Urine

Hydrolysis (0.33 ml. urine)	Added trigonelline	L value	Trigonelline recovered
	γ		γ
6 N KOH (+ urea)	0	0.164	
6 " " + "	8	0.190	2
5 " HCl followed by 6 N KOH (+ urea)	0	0.240	
" "	8	0.349	8.8

on all sorts of surfaces. It was for this reason that we avoid filtration through paper at any step in our proposed procedure, and use centrifugation instead.

From our adsorption studies it evolved that all of the nicotinic acid derivatives in concentrations dealt with in this work, and indeed all pyridine derivatives which were available to us, are quantitatively adsorbed on Lloyd's reagent at relatively high H ion concentrations, pH 1 or less. Furthermore, the adsorbed substances on the Lloyd's reagent can be washed repeatedly with dilute acid without any loss and can be recovered completely and readily by elution with dilute alkali solution. This observation proved to be of great advantage, in so far as it permitted us to isolate the pyridine derivatives from the large excesses of salts, inorganic acids, and of many organic substances.

It must be noted, however, that only a part of the pigments of the hydrolyzed or unhydrolyzed urine can be removed and washed out by this procedure; a relatively large proportion is adsorbed on Lloyd's reagent in acid and is eluted again by alkali. Of these residual pigments all but traces can be removed by the subsequent treatment with $Pb(NO_3)_2$ described above. We found, however, that 1 hour of boiling of the solids of 20 ml. of urine in 4 to 6 N HCl resulted in so large an amount of carbonaceous matter and of almost black (melanin-like ?) pigments that combining both of the above procedures with Lloyd's reagent and lead salts still yielded very dark colored solutions, and consequently high blank values. This led us to attempt to intensify the oxidizing action of the HCl by the addition of small amounts of nitric acid. The presence of 0.25 ml. of concentrated HNO_3 or of the corresponding amount of any nitrate yielded readily very clear solutions with relatively little color, most of which was removed by the subsequent Pb salt treatment. This amount, 0.25 ml., of concentrated HNO_3 is sufficient to oxidize the pigments without loss of nicotinic acid as measured by recovery, whereas the use of larger amounts of HNO_3 results in progressively increasing destruction of nicotinic acid.

The final solutions are not entirely colorless; a tinge of yellow always persists in the acid hydrolysate. The blank correction value due to this color is, under our conditions, relatively small. After the development of the nicotinic acid color in a 5 ml. sample of urine subjected to acid hydrolysis and containing 5 to 10 γ of nicotinic acid the absorption (L) value amounts to 0.120 to 0.250. The blank correction value for the same samples varies between 0.020 and 0.050, on the average 20 to 30 per cent of the color due to nicotinic acid. When the urine is not decolorized at all, as in the extrapolation procedure of Harris and Raymond (8), the blank value may amount to twice or 5-fold that due to nicotinic acid. In the original Bandier and Hald (4) procedure in which acetone extraction of the nicotinic acid is employed the blank color correction with urine is at least as great as the color due to the nicotinic acid. This is also true of the values given by Melnick and Field (9) for the only urine analysis quoted in their tables. It is obvious that a high correction value will tend to diminish the accuracy and reproducibility of results, even though

in a single run good recovery of added standard material may be secured. This is particularly true when, as in this case, the interfering pigments themselves react chromogenically with such active reagents as CNBr. We have not perfected a complete solution of this problem, but merely offer a procedure calculated to reduce this source of error to a minimum.

Color Reaction—The König reaction (15) for pyridine derivatives with CNBr and an aromatic amine has been employed by most of the recent authors for nicotinic acid determinations and

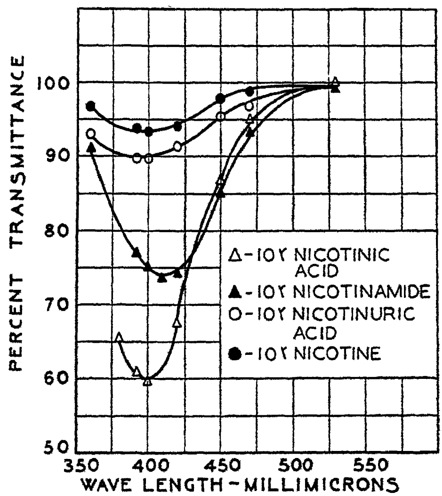

FIG. 1. Absorption curves of colors produced by the Bandier and Hald reaction with nicotinic acid derivatives and with nicotine. Coleman regional spectrophotometer, 15 mμ slit.

has been shown to be more suitable than the Vongerichten *p*-dinitrochlorobenzene reaction (16). After testing the various amines suggested by the previous investigators we have found the reaction with *p*-aminomethylphenol sulfate as employed by Bandier and Hald (4) to be the most desirable for the following reasons.

1. The color reaches a maximum in about 40 minutes and remains stable for several hours in the dark.

2. It is sensitive to nicotinic acid, 5 γ in our procedure giving an absorption value with the No. 400 filter of 0.105 to 0.115, and hence is easily determined. Bandier and Hald in reading the

color in a stufenphotometer use a No. S-43 filter with maximum transmission at 430 mμ. We found, as is seen in Fig. 1, that the absorption maximum of the nicotinic acid color is at 400 mμ. We have accordingly chosen the No. 400 Evelyn filter. We have thereby not only increased the sensitivity greatly but also gained reliability of values in reading the color at the minimum value for the slope of its absorption curve.

3. Other pyridine derivatives give a relatively low intensity of color with this reaction in the following approximate proportions: nicotinic acid 1.0, nicotinamide 0.5, nicotinuric acid 0.2, nicotine 0.1, and methyl pyridinium hydroxide 0.

These last relations make this reaction particularly suitable for the determination of the small amounts of nicotinic acid in the presence of the relatively large amounts of pyridine derivatives in smokers' urine. This is borne out by the following data.

Urine from a heavy smoker and from a tobacco chewer was evaporated and extracted exhaustively with benzene and the nicotinic acid derivatives determined by the above procedure before and after extraction. The figures obtained were 1.75 (smoker) and 1.1 γ per ml. (tobacco chewer) for unextracted samples and 1.70 (smoker) and 1.0 γ per ml. (chewer) for extracted samples. In both of these cases the benzene extracts were transferred to an aqueous acid phase and when tested by the CNBr aniline reaction showed the equivalent of 4 to 5 γ of nicotine per ml. Apparently this relatively large concentration of nicotine and of other pyridine bases extractable by benzene had practically no effect upon our nicotinic acid determination.

Table II summarizes our representative experiments showing recovery of nicotinic acid, nicotinamide, nicotinuric acid, and trigonelline added to urine as well as the recovery of these substances in pure solutions taken through our entire procedure. It is to be noted that the recovery of added nicotinuric acid is a more critical test than the recovery of nicotinic acid or of the amide because of the difficulty of its complete hydrolysis.

The following figures in terms of L values show the reproducibility of results with four samples of the same urine subjected to separate analyses: "total" nicotinic acid, 5 ml. of urine, 0.102, 0.106, 0.097, 0.103 (average 0.102); trigonelline, 0.33 ml. of urine, 0.324, 0.351, 0.316, 0.349 (average 0.335).

Daily Urinary Excretion of "Total" Nicotinic Acid and of Trigonelline by Normal Human Subjects

24 hour urines from five normal adults, four men and one woman, subsisting on an adequate diet, collected before and after

TABLE II

Recovery of Nicotinic Acid Derivatives

Hydrolysis	Urine	Added trigonelline	*L* values*		Recovered
			Urine	Urine + added substance	
	ml.	γ			γ
6 N KOH		20		0.252	20
5 " acid and 6 N KOH		20		0.248	20
5 " " " 6 " "	0.2	6	0.149	0.224	6
5 " " " 6 " "	0.2	12	0.149	0.299	12
5 " " " 6 " "	0.133	8	0.115	0.207	7.4
5 " " " 6 " "	0.133	16	0.115	0.290	14.0
		Nicotinic acid			
No hydrolysis		10		0.205–0.220	10
5 N acid		10		0.210	10
5 " "	5.0	6.25	0.088	0.217	6.2
5 " "	3.33	5	0.101	0.201	4.8
5 " "	2.66	2.5	0.054	0.110	2.6
		Nicotinamide†			
5 " "		10		0.210	10
5 " "	5.0	6.3	0.082	0.212	6.2
		Nicotinuric acid†			
5 " "		7.5		0.150	7.5
5 " "	5.0	7.5	0.107	0.240	7.0
5 " "	4.9	7.3	0.096	0.228	6.6

* The *L* value is the optical density obtained from the chart accompanying the Evelyn photoelectric colorimeter.

† The nicotinamide and the nicotinuric acid are given in terms of nicotinic acid.

the ingestion of doses of nicotinic acid, were analyzed by the method described above.

The content of the acid-hydrolyzable fraction, including free

nicotinic acid, the amide, and nicotinuric acid, is presented in Table III. It will be noted that the daily excretion of "total" nicotinic acid, exclusive of trigonelline, is remarkably constant in Subjects 2, 3, and 4 in whom it was studied several times, and that in all of the five subjects it varies from 1 to 3 mg. per day.

The data on the excretion of trigonelline in Subjects 2 to 5 are shown in Table IV in connection with the ingestion of doses of nicotinic acid. In distinction to the "total" nicotinic acid it was soon observed that the excretion of trigonelline was not only much greater, but also varied a great deal more in different individuals and in the same individual on different days. It was then

Table III

Daily Excretion of "Total" Nicotinic Acid in Five Subjects

Subject No.	Sex	Age	Weight	Mg. "total" nicotinic acid excreted per day on date indicated	Remarks
		yrs.	*lbs.*		
1	M.	48	240	Mar. 11, 3.0	Very heavy smoker
2	"	49	160	" 3, 1.6; June 25, 2.0; July 7, 2.2; July 25, 1.7; July 28, 1.8	Heavy smoker
3	"	25	155	Mar. 1, 1.5; May 22, 1.1; July 8, 1.4; July 19, 1.4; July 22, 1.2	Non-smoker
4	"	24	165	Mar. 3, 1.7; July 9, 1.4; July 11, 1.5; July 12, 1.4; July 23, 1.5	Moderate smoker
5	F.	19	145	July 25, 1.1	Non-smoker

suspected that trigonelline excretion bore a relation to dietary factors. No clear quantitative indication could be had from the data of Linneweh and Reinwein (13) who isolated trigonelline from the urines of subjects ingesting and abstaining from coffee, tea, and cocoa. However, the amount of trigonelline obtained from urine on the coffee-free diet was considerably less. Heiduschka and Brüchner (17) isolated 3 gm. of trigonelline from 2 kilos of defatted raw coffee beans. By subjecting several samples of freshly prepared black coffee beverage to our method of hydrolysis with strong alkali in the presence of urea we found that coffee, such as used by our subjects, may contain from 50 to 100 mg. per 200 ml. cup, depending upon its strength and pos-

sibly variety of bean, roasting, etc. It appears therefore that a coffee-drinking individual may ingest between 100 and 500 mg. of trigonelline per day in coffee alone. Trigonelline was also isolated in smaller amounts from various seeds such as peas, wheat, oats, hemp, and in potatoes (18, 19). Since coffee seemed to be the chief source of trigonelline, and since it was impractical to devise a totally trigonelline-free diet for human subjects, we repeated our experiments with our subjects remaining on the same general diet as before but omitting only coffee. This had an immediate and striking effect upon the daily excretion of trigonelline without affecting the other nicotinic acid derivatives.

TABLE IV

Changes in Daily Excretion Following Ingestion of Doses of Nicotinic Acid

Subject No.	Dose of nicotinic acid ingested	"Total" nicotinic acid excreted per day				Trigonelline excreted per day				Remarks
		Control	1st day	2nd day	3rd day	Control	1st day	2nd day	3rd day	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
2	100	2.2	3.3	2.0		204	203	116		Coffee
4	500		67.8				217			"
4	100	1.4	10.2	1.5	1.4	150	156	160	146	"
3	100		2.7	1.3	1.2		32	72	107	" irregularly
2	200	1.7	6.0	1.7	1.8	29	37	24	40	No coffee
4	200	1.6	19.4	1.5		20	44	36		" "
3	100	1.4	2.5	1.4	1.2	27	50	21	18	" "
5	200	1.1	12.5			25	36			" "

Thus, as seen in Table IV, Subject 2 excreted 116 to 204 mg. of trigonelline per day when ingesting coffee, and 24 to 40 mg. on a coffee-free diet. In general the same was true of the other three subjects. We also noted that apparently the trigonelline of the ingested coffee is promptly excreted, for 1 day's abstinence from coffee can bring down the excretion from around 200 mg. to 30 mg. per day.

The significance of the daily trigonelline excretion with or without the ingestion of extra nicotinic acid cannot yet be properly interpreted, since undoubtedly the coffee-free diet of our subjects still contained smaller but variable amounts of trigonelline. In

future experiments we shall attempt to select a constant diet with a low and a well defined trigonelline content.

It is obvious that until such a procedure is adopted it will not be possible to follow the metabolism of nicotinic acid in the human or animal body. In a purely qualitative way it can be noted that in each of the four subjects who ingested doses of 100 to 500 mg. of nicotinic acid in a single day when on a coffee-free diet there was a significant increase of the trigonelline excretion over that in the preceding or the following day.

As noted before, the "total" nicotinic acid excretion in our five subjects varied from 1.1 to 3 mg. per day. The response of four of these subjects in regard to "total" nicotinic acid excretion after the ingestion of 100 to 500 mg. doses of pure nicotinic acid was quite different. Whereas 100 and 200 mg. doses led to an increased "total" nicotinic acid excretion of only 1 to 4 mg. in two subjects (Nos. 2 and 3), the increases in the other two subjects (Nos. 4 and 5) were from 9 to 18 mg.

These individual variations in "total" nicotinic acid excretion seem to bear no proportional relation to the apparent increase in trigonelline excretion. In an effort to interpret these variations we attempted to identify the components of the acid-hydrolyzable or "total" nicotinic acid fraction. Since the free nicotinamide or that of the coenzyme complex is easily hydrolyzed by 1 hour's heating at 100° with 1 N HCl, and since this procedure results in the hydrolysis of only approximately 10 per cent of the nicotinuric acid, we determined the nicotinic acid color produced by the unhydrolyzed urine and by urine heated at 100° for 1 hour with 1 N HCl, using the 24 hour urines of Subjects 4 and 5 containing 19.4 and 12.5 mg. of "total" nicotinic acid respectively. Except for the hydrolysis the rest of our procedure was followed in all of these determinations. These urines were obtained on the day of ingesting 200 mg. of nicotinic acid by each subject. The results in terms of micrograms of nicotinic acid per ml. of urine were as follows:

Hydrolysis	Subject 4	Subject 5
Unhydrolyzed	3.5	2.6
1 N HCl	4.3	3.7
5 " $HCl-HNO_3$	12.9	10.4

It will be noted that hydrolysis with 1 N HCl led to but small increases in nicotinic acid, the large increases resulting from the 5 N acid hydrolysis. Since nicotinamide gives 50 per cent of its equivalent nicotinic acid color and is completely hydrolyzed by 1 N HCl, and, since nicotinuric acid gives 20 per cent of its equivalent nicotinic acid color, and is approximately 10 per cent hydrolyzed by 1 N HCl, it is obvious that in these urines the "total" nicotinic acid consisted largely of nicotinuric acid. Therefore, only small amounts of free nicotinic acid, nicotinamide, or coenzyme could have been present in these urines.

It thus appears that when the ingestion of doses of nicotinic acid is followed by appreciable increases of "total" nicotinic acid in the urine exclusive of trigonelline, such increases are largely due to the formation and excretion of nicotinuric acid. Since this substance can only be determined after its complete hydrolysis, and since such complete hydrolysis in urine without affecting the trigonelline can be accomplished, as far as is known, only by means of boiling with strong acid (5 to 6 N HCl) for at least 1 hour, it becomes important in saturation or tolerance tests to bear these relations in mind. Although the very low concentration of "total" nicotinic acid in normal urine (1 to 2 mg. per liter) makes it very difficult to determine the partition of its various forms, our data indicate that a complete hydrolysis with 5 N acid is necessary for its complete conversion and determination.

The data in the second part of Table IV on Subjects 2 to 5, after the ingestion of 100 or 200 mg. of nicotinic acid, show that (1) there are variable increases in the excretion of "total" nicotinic acid and of trigonelline on the day of the ingestion of the acid, returning to normal levels on the following day; (2) the sum of the increases in both fractions accounts for but 10 to 25 per cent of the ingested doses.

Granting that the interpretation of the trigonelline values is not wholly reliable because of the uncertainty of the constancy of intake with the food, it still does not appear that this factor when more properly controlled will account for the deficit, for if the total trigonelline excreted on these days were ascribed to the ingested nicotinic acid alone the figures would still fall far short of the ingested nicotinic acid. We are therefore led to conclude that ingested nicotinic acid, in 100 or 200 mg. doses, is excreted

promptly but only partially as nicotinuric acid and trigonelline. The proportion of these two derivatives varied in the four subjects widely. The fate of the rest of the ingested nicotinic acid can only be conjectured: it may in part be stored in the tissues, in part destroyed, not unlike ascorbic acid in this respect, and also possibly in part converted and excreted in forms which are yet not amenable to analytic detection; there is also the possibility of incomplete absorption and loss in the stools.

We intend to extend these studies on the metabolism of ingested nicotinic acid to include blood, urine, and tissue analyses in human subjects and in animals.

SUMMARY

A study of the nicotinic acid derivatives and trigonelline in normal human urine is presented.

Methods are proposed for determining the nicotinic acid derivatives after complete hydrolysis with strong hydrochloric acid and of estimating trigonelline after heating with strong alkali in the presence of ammonia. The difficulties due to interfering substances and to extraneous colors are discussed and methods for their circumvention are offered.

Five normal human adults on an adequate diet excreted daily 1 to 3 mg. of nicotinic acid derivatives exclusive of trigonelline. On a diet free of coffee they excreted 20 to 29 mg. of trigonelline per day and up to 200 mg. per day when ingesting coffee.

After the ingestion of 100 to 200 mg. doses of nicotinic acid four of the above subjects excreted 2.5 to 19.4 mg. of nicotinic acid derivatives, mostly as nicotinuric acid. The trigonelline excretion was appreciably increased, although the extent of this increase is not yet definitely established. These increases account for only 10 to 25 per cent of the ingested nicotinic acid.

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THE ENZYMATIC REDUCTION OF CYTOCHROME C

CYTOCHROME C REDUCTASE

BY ERWIN HAAS, B. L. HORECKER, AND T. R. HOGNESS

(*From the George Herbert Jones Chemical Laboratory of the University of Chicago, Chicago*)

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No enzyme or enzyme system has thus far been described which catalyzes the physiological reaction between cytochrome *c* and reduced triphosphopyridine nucleotide. Although several of the known flavoproteins react with reduced triphosphopyridine nucleotide none of them reacts directly with cytochrome *c*. Cytochrome *b* and hydrogen carriers presumably acting between cytochrome *c* and the flavoproteins have therefore been postulated as part of this scheme but no concrete evidence to support such postulated mechanisms has been presented. The reactions between the reduced forms of either Warburg and Christian's "old" (1) or Haas' "new" (2) yellow enzyme and oxygen are too slow to be physiologically important and these enzymes are therefore left without explanation of their enzymatic significance. Theorell (3) reported observations of the reduction of cytochrome *c* by the "old" yellow ferment but this reaction also was too slow for physiological consideration. In the attempt to bridge the gap in the oxidation-reduction chain, Straub (4) isolated a flavoprotein from heart muscle, but like the other flavoproteins this substance does not react with cytochrome *c* (5).

In this paper we report the isolation of a new flavoprotein[1] which, reacting very rapidly with both oxidized cytochrome *c*

[1] In a preliminary report on the isolation of this enzyme we described it as colorless. During the first stages of its isolation the product lost much of its color upon purification. Judging from the activities of the previously isolated yellow ferments, we were led to the conclusion that the product could not be as active as it was and still show no yellow color. We were, therefore, misled by the extremely high activity of this enzyme.

and reduced triphosphopyridine nucleotide, completes the oxidation-reduction system from hexose monophosphate to cytochrome c. The prosthetic group of this new enzyme is alloxazine mononucleotide.

According to the nomenclature suggested by Warburg this enzyme could be designated as

alloxazine-proteid $_{\text{cytochrome } c\text{ - triphosphopyridine nucleotide}}$

We shall refer to it as cytochrome c reductase.

General Principles of Analytical System

The whole enzyme system which catalyzes the reduction of cytochrome c by hexose monophosphate is made up as follows: hexose monophosphate, *Zwischenferment*, triphosphopyridine nucleotide, cytochrome c reductase, cytochrome c. The triphosphopyridine nucleotide is rapidly reduced, while the oxidized cytochrome c does not react until the reductase is finally added. The concentrations of all components are so adjusted that (1) the rate of reduction of the cytochrome c is proportional to the reductase concentration and (2) the half time of the cytochrome c reduction is about 3 minutes. Each of the components of the system is sufficiently pure so that no reaction proceeds until the cytochrome c reductase is added.

Preparation of Test Substances

Triphosphopyridine Nucleotide—This substance was prepared by a modification of the method described by Warburg, Christian, and Griese (6). We are indebted to Dr. T. S. Ma for the following analysis of the final product.

$C_{22}H_{32}N_7P_3O_{19}$.	Calculated.	C 33.4, H 4.06, P 11.8
	Found.	" 34.8, " 4.37, " 9.3

On the basis of the phosphorus content we calculate a purity of 79 per cent for the triphosphopyridine nucleotide.

Using the manometric test for triphosphopyridine nucleotide (6) we found a purity of 80 per cent for our product.

Hexose Monophosphate—This was prepared by a modification of the directions given by Robison and Morgan (7) and by Warburg and Christian (1). We have found that bottom yeasts are

unsatisfactory for this preparation. Top ale yeasts, however, gave excellent results.

The best results are obtained when the Lebedew extract is prepared by digestion at 0° rather than at 35° as specified by Warburg and Christian. The yeast used must be fresh and it should be washed immediately after the brewing process. The extract should be protected from air during the extraction.

The yeast used in the preparation of the hexose monophosphate was the same as that used as the source of the reductase. 900 gm. of the dried powdered yeast were stirred into 2700 cc. of water and extracted for 22 hours at 0°. The air in the covered container was displaced by carbon dioxide. Centrifugation and filtration yielded 1.4 liters of the clear Lebedew extract, which was used at once for the preparation of hexose monophosphate. From 300 gm. of glucose, 300 gm. of $Na_2HPO_4 \cdot 12H_2O$, and 20 gm. of KH_2PO_4 we obtained 42 gm. of the crystalline calcium salt of hexose monophosphate.

Analysis of this material by Dr. T. S. Ma gave the following result.

3.128 mg. sample yielded 19.70 mg. ammonium phosphomolybdate
3.416 " " " 1.240 " H_2O and 2.784 mg. CO_2
3.681 " " " 1.403 " " " 2.986 " "

Calculated, $C_6H_{11}O_5CaPO_4 \cdot H_2O$.	H 4.11,	C 22.8,	P 9.8
Found (average).	" 4.13,	" 22.18,	" 9.15

The calcium salt was converted into the potassium salt by treatment with potassium oxalate. 3.15 cc. of M oxalate were necessary for 1 gm. of the calcium salt. Thus 1 mole of calcium salt (mol. wt. 316) was equivalent to 0.995 mole of potassium oxalate.

Zwischenferment—The specific protein which is necessary in the reduction of triphosphopyridine nucleotide by hexose monophosphate was prepared in accordance with the directions given by Warburg and Christian (1). This is a relatively impure product but contained no cytochrome reductase, whereas the purest *Zwischenferment* obtained by the method of Negelein and Gerischer (8) contained some of the reductase.

Canadian top ale yeast which was used to make this preparation was washed and dried. A suspension of 400 gm. of the dried yeast in 1200 cc. of water was allowed to stand for 10 hours at

35°. The suspension was then centrifuged and the *Zwischenferment* precipitated from the supernatant solution by dilution with water and subsequent saturation with carbon dioxide. The precipitate, dried *in vacuo* over P_2O_5, gave 6.4 gm. of a dry powder which has not lost its activity in more than 1 year's time. 0.1 mg. of this powder was necessary for each test.

Cytochrome c—Following the directions of Keilin and Hartree (9), we obtained from 5.8 kilos of horse heart 3.1×10^{-5} mole of cytochrome. The concentration was determined spectrophotometrically. Using the absorption coefficients given by Theorell (10), we found 5.17×10^{-7} mole of cytochrome per cc., while for the same solution, after being reduced, 5.15×10^{-7} mole per cc. of cytochrome was found. This result showed that the solution was free from colored impurities.

Isolation of Enzyme

The activity of an enzyme preparation was tested spectrophotometrically by the method to be described in detail in the next section. The protein concentrations were determined by evaporating the salt-free solutions. In the presence of salt the solutions were subjected to micro-Kjeldahl determinations for protein nitrogen. The protein concentrations were calculated on the assumption that all proteins present contained 16 per cent nitrogen.

The activity of each preparation is here calculated on the basis of mg. of dry protein. This activity (W) is defined as follows:

$$W = \frac{\Delta \text{ log oxidized cytochrome } c \text{ concentration}}{\Delta t \times \text{mg. protein}}$$

W is independent of the cytochrome c concentration. For the pure enzyme W has a value of 158 mg.$^{-1}$ min.$^{-1}$.

Source Material—Top ale yeast of the strain *Saccharomyces cerevisiae* I, Hanson, obtained from Drewry's, Ltd., was washed at 0° with tap water and then subjected to high pressure to remove the excess water. By blowing air over a thin layer of the yeast for a few hours it was thoroughly dried. The dried yeast can be stored at room temperature without apparent loss of activity.

Step 1. Extraction of Enzyme by Autolysis—4 kilos of the dry

yeast are suspended in 14 liters of water and kept for 33 hours at 20°. The suspension is centrifuged and 6.9 liters of clear solution obtained which contain most of the enzyme. The residue is washed with 6.5 liters of water and centrifuged. The resulting supernatant solution is combined with the original to give 13.8 liters of an enzyme solution which contained 1400 gm. of dry substance. $W = 0.27$ mg.$^{-1}$ min.$^{-1}$. Since W for the pure enzyme is equal to 158 mg.$^{-1}$ min.$^{-1}$, the purity of the enzyme in the first extract is $0.27/158 = 0.0017$. The enzyme is very easily denatured and the whole of the following procedure must therefore be carried out at 0°.

Step 2. Fractionation with Ammonium Sulfate—At pH 4.5 the enzyme is soluble when the solution is 31 per cent saturated with ammonium sulfate. Under these conditions a large amount of inert proteins is denatured and can be removed. The enzyme is precipitated when the solution is made 51 per cent saturated with respect to ammonium sulfate.

Specifically, 13.8 liters of enzyme solution are cooled to 0°. While the solution is being stirred, 4.2 kilos of solid ammonium sulfate and then 220 cc. of 10 N acetic acid are added. The saturation is then 51 per cent with respect to ammonium sulfate, pH 4.5. The precipitate is separated by centrifugation. It contains the enzyme and can be kept in this form overnight. The precipitate, which has a volume of 1.1 liters, is suspended in 0.7 liter of water and 3 liters of a solution 31 per cent saturated with ammonium sulfate are added. The insoluble material is separated by centrifugation and discarded.

From the supernatant solution (4 liters) the enzyme is precipitated by adding 470 gm. of ammonium sulfate; the degree of saturation is then 51 per cent. After separation by centrifugation the enzyme is dissolved in 500 cc. of water. The solution contains 87 gm. of dry substance, other than ammonium sulfate. $W = 1.92$; purity after step (2) = 0.012; purification 7-fold; yield 44 per cent.

Step 3. Dialysis—For the following precipitation with alcohol it is necessary to remove the dissolved ammonium sulfate. Consequently the enzyme is dialyzed in cellophane tubes against running distilled water for 17 hours at 0°. After the removal of much inactive precipitate by centrifugation, 1200 cc. of enzyme

solution containing 52 gm. of protein are left. $W = 2.0$; purity $= 0.0126$; purification 1.06-fold; yield 63 per cent.

Step 4. Precipitation with Ethanol—1200 cc. of dialyzed enzyme solution are adjusted to pH 4.65 by adding 1.5 cc. of 2 N KOH. Slowly and with stirring in an ice bath, 600 cc. of 30 per cent cold ethanol are added. A fine white precipitate is formed which contains the enzyme. After 45 minutes the suspension is centrifuged and the precipitate is dried as rapidly as possible *in vacuo* over $CaCl_2$ at 0°. After this step 5.0 gm. of a dry powder are obtained. $W = 12$; purity $= 0.076$; purification 6-fold; yield 58 per cent. In 34 days 9 per cent of the activity of the dry powder is lost when kept at 0° *in vacuo* over $CaCl_2$.

Step 5. Adsorption on Aluminum Hydroxide Gel; Elution with Alkaline Ammonium Sulfate—5.0 gm. of the enzyme powder are dissolved in 200 cc. of water. By adding 5 cc. of N KOH the pH is adjusted to about 9. Aluminum hydroxide gel γ prepared according to Willstätter and Kraut (11) is added fractionally until the supernatant solution is just colorless. Excess adsorbent should be avoided. About 7.0 gm. of aluminum hydroxide are usually necessary to adsorb all the enzyme.

The enzyme is eluted from the aluminum hydroxide by adding 50 cc. of a solution which is 64 per cent saturated with ammonium sulfate and is 0.1 N with respect to NH_4OH. This elution with alkaline ammonium sulfate is repeated twice. From the combined eluates (150 cc.) the enzyme is precipitated by adding 18 cc. of 2 N acetate buffer, pH 4.5, and 30 gm. of solid ammonium sulfate (degree of saturation, 70 per cent). The precipitate is centrifuged for 1 hour at 3000 times gravity to remove as much as possible of the ammonium sulfate solution which interferes in the following step. The enzyme is dissolved in 100 cc. of water and 4 cc. of N NH_4OH are added to adjust the pH to about 9. The solution now contains 360 mg. of protein. $W = 51$; purity $= 0.32$; purification 4.2-fold; yield 30 per cent.

Step 6. Adsorption on Calcium Phosphate; Elution with Phosphate—Tricalcium phosphate gel was prepared by following the direction of Utkin (12) and was used about 8 months after preparation. The tricalcium phosphate is added to the enzyme solution in small portions until the supernatant solution remains colorless. At this particular salt concentration about 6.5 gm. of

calcium phosphate are sufficient for adsorption of the enzyme. The tricalcium phosphate with the adsorbed enzyme is washed with 80 cc. of 0.02 M borate buffer, pH 9.2, and the enzyme is eluted with 40 cc. of 0.05 M phosphate buffer, pH 6.1. The elution is repeated two times and the eluates are combined. Excess of phosphate, which interferes in the next purification step, should be avoided. The 120 cc. of the phosphate solution contain 44 mg. of protein. W = 98; purity = 0.62; purification 1.95-fold; yield 24 per cent.

Step 7. Adsorption on Aluminum Hydroxide Gel Repeated—120 cc. of the phosphate solution are stirred for 10 minutes with 1.7 gm. of aluminum hydroxide and centrifuged. The colorless supernatant solution is discarded. The enzyme is eluted from the aluminum hydroxide three times with 12 cc. of alkaline ammonium sulfate as in step (5). To the 36 cc. of the eluate 7.5 gm. of solid ammonium sulfate, and 4 cc. of 2 N acetate buffer, pH 4.5, are added. The degree of saturation with ammonium sulfate is 70 per cent. The precipitated enzyme is separated by centrifugation and dissolved in water. With 0.2 cc. of N NH_4OH the pH of the solution is adjusted to about 9. 17 mg. of enzyme dissolved in 6.2 cc. of solution are finally obtained. This solution is 0.2 M with respect to $(NH_4)_2SO_4$ but since a dilution of 1:7000 is made for the test there is no interference. W = 138; purity = 0.87; purification 1.41-fold; yield 55 per cent.

The titration with cytochrome as given in the next section proves that the enzyme after step (7) is 87 per cent pure. Although kept at 0°, the pure enzyme loses 30 per cent of its activity in 2 days time. Considering the instability of the enzyme, we believe that most of the inactive substance is cytochrome *c* reductase, denatured during the purification process.

Remarks Pertinent to Isolation—We have found cytochrome reductase in yeast obtained from different sources. The best results in the isolation are obtained with a yeast which autolyzes readily at low temperatures.

During the course of the isolation the purity of the enzyme increases from 0.0017 for the first step to 0.87 for the last step, an increase of about 500-fold. Assuming that the first extract contained all of the enzyme, we calculate that 1 kilo of dry yeast contains about 0.6 gm. of the enzyme (0.0017 × 1400/4).

By the same method of calculation the amount of "old" yellow enzyme in Warburg and Christian's original source and the amount of "new" yellow enzyme in that of Haas have been calculated. These are here compared with the amount of cytochrome reductase found in our yeast (Table I).

It is interesting to note that the concentration of cytochrome reductase and that of the "old" yellow ferment are of the same order of magnitude.

The over-all yield for the seven steps of the isolation is equal to 0.63 per cent. By this process of purification about 235 kilos of dry yeast (approximately 1 ton of fresh yeast) would be necessary to obtain 1 gm. of the enzyme.

TABLE I

Yellow Enzymes in Yeast

Enzyme	Concentration, gm. enzyme per kilo dry yeast	Source material
Cytochrome *c* reductase....	0.6	Drewry's ale yeast
"Old" yellow enzyme .	1.0	Schultheiss-Patzenhofer yeast
"New" " "	0.14	" " "

Purity of Final Product and Molecular Weight of Enzyme

In calculating the purity of the final product and in estimating the molecular weight we compare its properties with those of Warburg and Christian's "old" and Haas' "new" yellow enzyme.

Absorption Spectrum—The absorption spectrum from 250 to 550 mμ is given in Fig. 1, and the more detailed spectrum in the characteristic range 320 to 550 mμ is given in Fig. 2. The absolute values for the extinction coefficients[2] (ordinates) have been calculated with the assumption that the value at 455 mμ for cytochrome reductase is 1.04×10^7 cm.^{2} mole^{-1}. This is the extinction coefficient of the "old" yellow enzyme (13) at 465 mμ, of

[2] The extinction coefficient is defined by α in the relationship $I = I_0 10^{-\alpha c l}$ where c is expressed as moles per cc. The absorption coefficient β is defined by the equation $I = I_{0e}{}^{-\beta c l}$. The value of β for the wave-lengths 455 mμ is then 2.4×10^7 cm.^{2} $\times$ mole^{-1}.

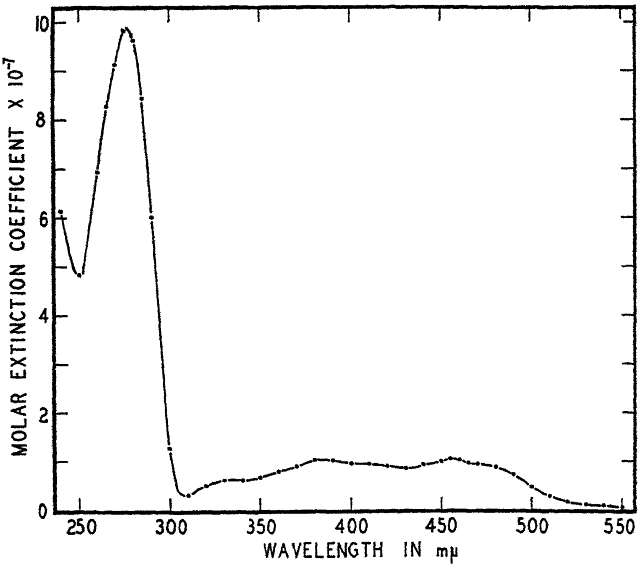

FIG. 1. Absorption spectrum of cytochrome *c* reductase

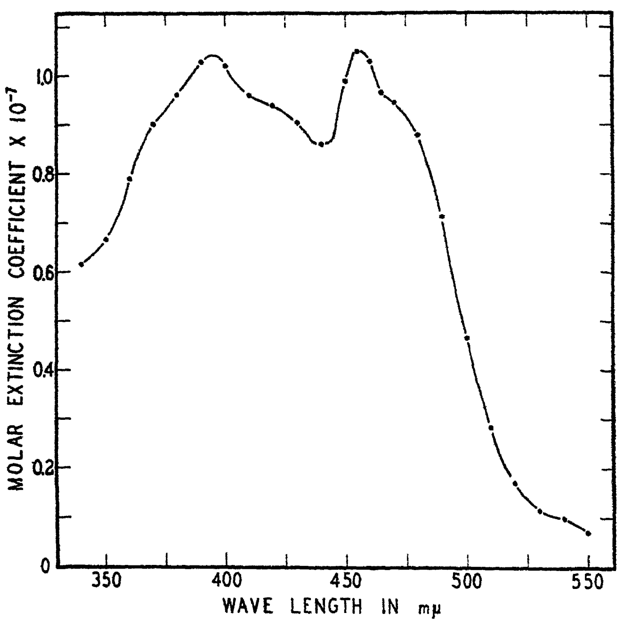

FIG. 2. Absorption spectrum of cytochrome *c* reductase, near ultraviolet and visible regions.

the "new" yellow enzyme (2) at 455 mμ, and of the amino acid oxidase (14) at 450 mμ.

The absorption spectrum of cytochrome reductase is made up of the three bands which are typical for the yellow enzymes, but as Table II indicates these bands are not identical with those of either the "old" or "new" enzymes.

The slight inflection in the absorption curve between 400 and 450 mμ indicates that a band at about 420 mμ may also be present. That this inflection is due to an impurity, very probably a hemin compound, was demonstrated by the fact that a band at this wave-length persisted when the cytochrome c reductase was reduced with the hexose monophosphate system. From the magnitude of this band we estimate that the maximum amount of hemin protein was 3 per cent. A small band also appeared at 557

TABLE II

Absorption Maxima of Yellow Enzymes

Enzyme	λ_1	λ_2	λ_3
	mμ	*mμ*	*mμ*
Cytochrome reductase.............	275	385	455
"Old" yellow enzyme.............	275	380	465
"New" " "	275	377	455

mμ. Although we have reason to believe this substance to be an impurity, its properties will be investigated further.

Determination by Light Absorption—With the assumption (1) that the extinction coefficient at the wave-length 455 mμ for cytochrome c reductase is 1.04×10^7 cm.^{2} mole^{-1} and (2) that the enzyme is 100 per cent pure, we may calculate its molecular weight.

The enzyme solution obtained from step (7) in the isolation contained 2.75 mg. of protein per cc. For the same solution the light absorption, I_0/I, at 455 mμ was found to be 1.555 in an absorption cell 0.504 cm. in length. The molecular weight was then calculated in the following manner.

$$C \text{ (flavin)} = \frac{1}{\alpha \times l} \log \frac{I_0}{I} = \frac{0.192}{1.04 \times 10^7 \times 0.504} = 3.66 \times 10^{-8} \text{ mole per cc.}$$

If 1 mole of the enzyme contains 1 mole of flavin, then the calculated molecular weight is $(2.75 \times 10^{-3})/(3.66 \times 10^{-8})$ or 75,000.

Using a method similar to that employed here, Theorell (13) found a molecular weight of 73,000 for the "old" yellow enzyme. Kekwick and Pedersen (15) using the ultracentrifuge found by the equilibrium method a molecular weight of 82,800 for this same enzyme, while Polson (16) determined the molecular weight to be 77,800 by the sedimentation method. We can assume that within the limits of all the experimental errors the molecular weights of these two enzymes have the same value. In the following calculations we shall therefore assume the molecular weight of cytochrome *c* reductase to be 78,000.

From these same considerations we would arrive at the conclusion that our final product is a pure one but such a conclusion would be based on the assumption that the only yellow enzyme contributing to the light absorption was cytochrome *c* reductase. To determine its purity in this respect reduced cytochrome *c* reductase was titrated with cytochrome *c*.

Titration with Cytochrome c—Reduced cytochrome *c* reductase reacts with oxidized cytochrome *c* ($CyFe^{+++}$) according to the equation

$$\text{Reduced cytochrome } c \text{ reductase} + 2CyFe^{+++} = \text{cytochrome } c \text{ reductase} + 2\,CyFe^{++} + 2H^{+}$$

Hexose monophosphate, *Zwischenferment*, and triphosphopyridine nucleotide were added to a solution containing the reductase. Excess hexose monophosphate was used but the amounts of *Zwischenferment* and triphosphopyridine nucleotide were such that the reduction took a relatively long time. After the reductase was reduced, an excess of oxidized cytochrome *c* was added. With relatively large concentrations the reaction between these constituents took place immediately and the amount of the cytochrome *c* reduced was determined spectrophotometrically at 550 mμ. After the initial instantaneous reduction, the excess cytochrome *c* was reduced but this subsequent reduction was slow because of the limiting amount of *Zwischenferment*.

Determinations were made with two enzyme concentrations

TABLE III

Titration of Enzyme

λ = 550 mμ, total volume = 5.8 cc., length of cell = 1.98 cm.; gas space, nitrogen.

	Sample 1	Sample 2
0.025 M phosphate buffer, pH 7.3....	5.8 cc.	→
Hexose monophosphate....... ...	2.5 mg.	
Triphosphopyridine nucleotide.....	0.007 mg.	
Zwischenferment..................	0.80 "	
Cytochrome reductase.............	0.52 "	1.04 mg.
Oxidized cytochrome c (added later)	4.7×10^{-8} mole	4.7×10^{-8} mole
Cytochrome reduced initially.......	1.18×10^{-8} "	2.32×10^{-8} "

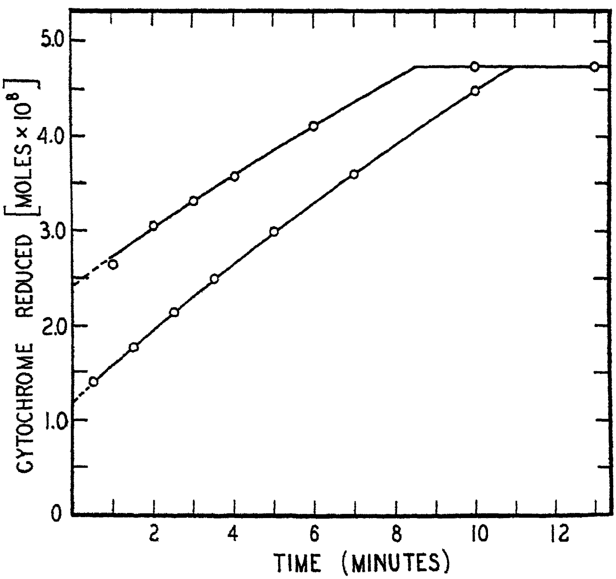

FIG. 3. Titration of reduced cytochrome c reductase, by oxidized cytochrome c. Upper curve 1.04 mg. of enzyme; lower curve 0.52 mg. of enzyme.

with identical results. The conditions under which these titrations were made are given in Table III and the results are shown in Fig. 3.

By extrapolating the curves to zero time, the amount of cytochrome c reduced instantaneously is obtained. The number of

moles of cytochrome *c* reduced in each case is given in the last row of Table III. Since 2 moles of cytochrome *c* are equivalent to 1 mole of cytochrome *c* reductase, we find that Sample 1 contains 0.59×10^{-8} mole of the reductase and Sample 2 contains 1.16×10^{-8} mole.

Assuming a molecular weight of 78,000 for the enzyme, Sample 1 contains $0.59 \times 10^{-8} \times 78{,}000 \times 10^{3} = 0.46$ mg. of the active enzyme, while Sample 2 contains 90 mg. The purity of the enzyme preparation is given by the relation, (active enzyme)/(total protein) = 0.46/0.52 = 0.87 for Sample 1 and 0.90/1.04 = 0.88 for Sample 2.

On the basis of this titration and of the flavin determination in the previous section, we conclude that the enzyme was about 87 per cent pure and that inactive yellow enzyme accounted for most of the remainder.

The fact that identical results were obtained when different amounts of the reactants were used shows that the reaction goes to completion.

Stability of Enzyme

At pH 8.8 and at 0° the purest product in a 0.05 saturated $(NH_4)_2SO_4$ solution lost 30 per cent of its activity in 2 days. At pH 4.5 the enzyme is still less stable, losing as much as 50 per cent activity in 15 hours. When the enzyme solution was frozen by evaporation and then dried *in vacuo*, the dry powder lost only 30 per cent of its activity in 26 days if kept *in vacuo* over $CaCl_2$ at 0°.

The activity of the purified enzyme is destroyed by dialysis against distilled water. When dialyzed against 0.001 M NH_4OH, the enzyme is stable.

The inactivation of the enzyme by heat depends very greatly upon the salt concentration of the solution and upon the purity of the product.

At pH 7.3 in 0.06 M $(NH_4)_2SO_4$ the purest enzyme loses activity at the following rate: in 10 minutes, at 30° 19 per cent, at 40° 37 per cent, and at 45° 58 per cent.

The activity is destroyed by acetone and by dioxane.

Rudimentary Kinetics and Enzyme Test

Since the whole oxidation-reduction system consists of a series of steps, the rate of reduction of the cytochrome *c* may depend

upon the rate of any of these steps. We have not as yet separated these steps in making rate determinations; yet enough data are at hand to give minimum values for the rate constants of the reactions involving the oxidized and reduced forms of cytochrome c reductase. Under the conditions prevailing in our analytical experiments the rate of reduction is proportional to the concentration of the cytochrome c reductase. Since the reduction of oxidized cytochrome c involves the reduced form of the reductase, we may assume that all of the enzyme is present in the reduced form. Denoting oxidized cytochrome c by $CyFe^{+++}$ we can express the over-all rate by the equation

$$-\frac{d(\mathrm{CyFe^{+++}})}{dt} = K(\mathrm{CyFe^{+++}})(\text{reduced cytochrome } c \text{ reductase}) \qquad (1)$$

or

$$-\frac{d \log_{10}(\mathrm{CyFe^{+++}})}{dt} = \frac{K}{2.3}(\text{reduced cytochrome } c \text{ reductase}) \qquad (2)$$

Under these conditions the concentration of the reduced reductase remains constant during the course of the reaction and d log $(\mathrm{CyFe^{+++}})/dt$ is constant. This is demonstrated by the data given in Table IV.

The data given in Table IV are those used for determining the activity of our purest sample. All constituents except the cytochrome c reductase were added to the cell. After a few minutes were allowed for the triphosphopyridine nucleotide to become completely reduced, 0.00042 mg. of the reductase was added. The galvanometer reading was taken before adding the reductase and every minute thereafter. The value of the enzyme activity, W, for the final product was calculated to be 138 min.$^{-1}$ $\times$ mg.$^{-1}$.

$$W = \frac{\Delta \log(\mathrm{CyFe^{+++}})}{\Delta t \times \text{mg. enzyme}} = \frac{0.058}{0.00042} = 138$$

By plotting the logarithm of the oxidized cytochrome concentration against the time of the reaction a straight line should be obtained and if Equation 2 is true the slope of the line should be proportional to the enzyme concentration. This plot is shown in Fig. 4 for three reactions with different reductase concentrations. By plotting the slopes of these curves against enzyme

concentration a straight line is obtained, in agreement with Equation 2 (Fig. 5).

The above considerations form the basis for the analytical test for the reductase. Under these conditions the rate of reduction is proportional to the enzyme concentration. A more thorough

TABLE IV

Test for Enzyme Activity

I_0 = light intensity (in arbitrary units) after passing through blank cell containing no cytochrome.

I = light intensity after passing through cell containing the cytochrome.

l = length of cell = 0.32 cm.

λ = 550 mμ.

α oxidized (for $CyFe^{+++}$) = 0.0956×10^8 cm.^{2} $\times$ moles^{-1}.

α reduced (for $CyFe^{++}$) = 0.281×10^8 cm.^{2} $\times$ moles^{-1}.

C = total cytochrome concentration = 5.4×10^{-8} mole per cc.

$$(CyFe^{+++}) = \frac{1/l \log I_0/I - \alpha \text{ reduced} \times C}{\alpha \text{ oxidized} - \alpha \text{ reduced}}.$$

Temperature 25°; gas space, air.

The following constituents were added, 1.0 cc. of 0.025 M phosphate buffer, pH 7.3, 0.90 mg. of potassium salt of hexose monophosphoric acid, 0.10 mg. of *Zwischenferment*, 0.020 mg. of triphosphopyridine nucleotide, and 0.86 mg. of cytochrome *c*.

Time	Galvanometer reading I_0	Galvanometer reading I	$\frac{I_0}{I}$	$(CyFe^{+++})$	Log $(CyFe^{+++})$ + 8	$\Delta \log (CyFe^{+++})$	$\frac{\Delta \log (CyFe^{+++})}{\Delta t}$
min.				*moles per cc.* $\times 10^8$			*min.*$^{-1}$
0	180	123	1.47	5.40	0.732		
Added 0.00042 mg. reductase							
1	180	111	1.62	4.70	0.672	0.060	0.060
2	180	103.5	1.74	4.14	0.617	0.115	0.058
3	180	96	1.87	3.62	0.559	0.173	0.058
4	180	90	1.99	3.16	0.500	0.232	0.058
5	180	85.5	2.10	2.76	0.440	0.292	0.058
6	180	80.5	2.23	2.36	0.373	0.359	0.060

study of the kinetics of this system will be presented in a later report.

The reaction between cytochrome *c* and cytochrome *c* reductase is first order with respect to each of these constituents. Since the cytochrome *c* undergoes a 1 valence change and the reductase a

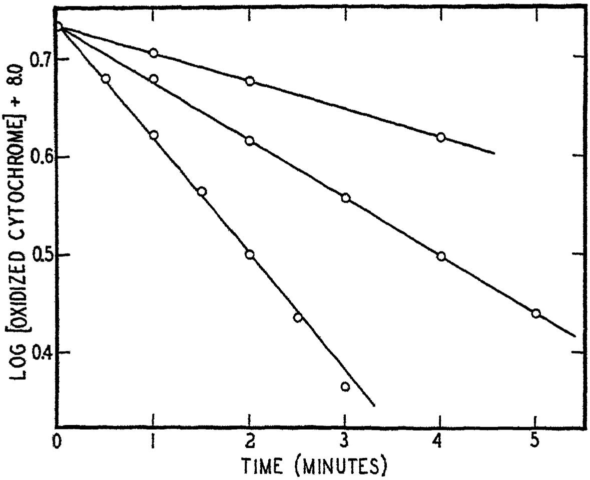

FIG. 4. Rate of reduction of cytochrome *c*. Top curve, 0.21 γ of reductase; middle curve, 0.42 γ; bottom curve, 0.84 γ.

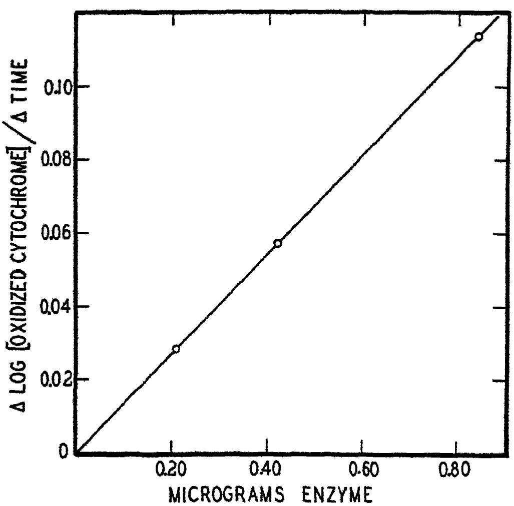

FIG. 5. Rate of reduction of cytochrome *c* as a function of reductase concentration.

2 valence change, we may tentatively postulate that the reductase forms an intermediary semiquinone-like free radical in the course of the reaction. This conclusion is not surprising in view of the finding of Haas that such a compound was observable for the "old" yellow enzyme (17).

From the data in Table IV we are able to calculate minimum values for the specific reaction velocities for the oxidation of cytochrome *c* reductase by cytochrome *c* and also for the reduction of the reductase by dihydrotriphosphopyridine nucleotide. In the first case we assume that all the reductase is in the reduced form; in the second case we assume that the reductase is all in the oxidized form. As these values for the oxidized and reduced

TABLE V

Specific Reaction Velocities at 25°

K = min.$^{-1}$ mole^{-1} liter.

Enzyme	Prosthetic group	Reaction with dihydrotriphosphopyridine nucleotide, K (reduced) $\times 10^{-5}$	Reaction with cytochrome *c*, K (oxidized) $\times 10^{-5}$	Reaction with oxygen, K (oxidized) $\times 10^{-5}$
Cytochrome *c* reductase	Alloxazine mononucleotide	>520	>280	
"Old" yellow enzyme	" "	60	0.3	1
"New" yellow enzyme	Alloxazine dinucleotide	220	0	0.14

concentrations of the reductase are maximum values, we obtain only minimum values for the reaction constants. In Table V we compare these values with the known or calculable values for reactions involving the "old" and the "new" yellow enzymes (2). The constant for the reaction between "old" yellow enzyme and cytochrome *c* is based upon data obtained with our own preparation. We have also included the specific reaction velocity constants for the reactions between the "old" and "new" yellow enzymes and oxygen in order to show the relative rates of oxidation by cytochrome and by oxygen. We have not yet determined whether the reduced form of our enzyme can be readily oxidized by oxygen but the evidence is definite that this enzyme is much

more active toward triphosphopyridine nucleotide and cytochrome *c* than either of the other two enzymes. The reduction of cytochrome *c* by the "old" yellow enzyme as reported by Theorell (3) can easily be explained by the assumption that preparations of this enzyme contained only 1 part per thousand of active cytochrome *c* reductase.

Prosthetic Group

In the following we shall demonstrate that the prosthetic group of cytochrome *c* reductase is alloxazine mononucleotide, the same prosthetic group as that associated with the "old" yellow enzyme. To do this we shall describe experiments in which the prosthetic groups of cytochrome *c* reductase, of the "old" yellow enzyme, and of the amino acid oxidase are interchanged. We shall also substantiate our conclusion by a report of a phosphorus determination on the prosthetic group of the reductase.

Splitting and Resynthesis of Cytochrome c Reductase

The reductase can be split and separated into a flavin and a colorless protein in the presence of a high hydrogen ion concentration. The method of effecting this splitting was first suggested by Warburg and Christian (18). Each part of the dissociated enzyme is by itself inactive but by combining the two the active enzyme can be resynthesized.

The prosthetic group was prepared by dissolving 70 mg. of enzyme (obtained from step (5), purity = 0.32) in 8 cc. of water at 0°. To this solution were added 2 gm. of $(NH_4)_2SO_4$ and 2 cc. of N H_2SO_4. The pH of the resulting solution was 2.3 and the degree of saturation was 0.35 with respect to $(NH_4)_2SO_4$. The precipitated protein was separated by centrifugation and discarded. The supernatant solution which contained the flavin was neutralized with 0.50 cc. of 2 N KOH. We thus obtained 9.0 cc. of a clear yellow solution. The flavin in this solution we shall designate henceforth as flavin$_{c.r.}$. Spectrophotometrically this solution was found to contain 0.40×10^{-7} mole of flavin per cc.

To obtain the active protein component 8.8 mg. of an enzyme preparation (purity = 0.62) were dissolved in 6 cc. of a solution which was 35 per cent saturated with $(NH_4)_2SO_4$, at 0°. 0.8 cc. of a solution which was 35 per cent saturated with $(NH_4)_2SO_4$

and was 0.1 N with respect to H_2SO_4 was then added so that Congo paper was just blue. The protein was precipitated and separated by centrifugation. It was then washed with 3 cc. of a 50 per cent saturated $(NH_4)_2SO_4$ solution and dissolved in 4 cc. of 0.05 M phosphate buffer, pH 7.3. 1 cc. of this colorless solution contained 1.5 mg. of protein which we shall designate as protein$_{c.r.}$; purity 0.50, yield 37 per cent.

The activity of each of the components and of the resynthesized enzyme was determined by means of the test previously described

TABLE VI

Enzymatic Activity of Separated and Combined Enzyme Components

Protein$_{c.r.}$ = 0.92 γ per cc.			Flavin$_{c.r.}$ = 2.5 × 10^{-11} mole per cc.			Protein$_{c.r.}$ = 0.92 γ per cc. Flavin$_{c.r.}$ = 2.5 × 10^{-11} mole per cc.		
t	8 + log ($CyFe^{+++}$)	$\frac{\Delta \log (CyFe^{+++})}{\Delta t}$	t	8 + log ($CyFe^{+++}$)	$\frac{\Delta \log (CyFe^{+++})}{\Delta t}$	t	8 + log ($CyFe^{+++}$)	$\frac{\Delta \log (CyFe^{+++})}{\Delta t}$
min.			*min.*			*min.*		
0	0.710		0	0.734		0	0.720	
1	0.698	0.012	1	0.731	0.003	1	0.633	0.087
2	0.684	0.013	2	0.728	0.003	1.5	0.606	0.076
						2	0.570	0.075
Average for $\frac{\Delta \log(CyFe^{+++})}{\Delta t}$ = 0.012			0.003			0.079		

Protein$_{c.r.}$ = protein of cytochrome reductase; flavin$_{c.r.}$ = flavin of cytochrome reductase.

(Table IV). In Table VI the activities of flavin$_{c.r.}$, protein$_{c.r.}$, and flavin$_{c.r.}$ + protein$_{c.r.}$ are compared.

This experiment shows clearly that the activity is restored when the two components are recombined. The small activity of the protein alone is probably due to some unsplit enzyme in the protein solution.

Dissociation Constant

To determine the constant for the dissociation of cytochrome *c* reductase into the prosthetic group and protein we added the

flavin in different concentrations to a small but constant amount of the protein at pH 7.3 and at 25°. The activity of the resynthesized enzyme was determined as usual (Table IV). For these determinations only 0.9 γ of protein and about 3×10^{-3} γ of flavin are necessary; this makes available a method for determining very small amounts of alloxazine mononucleotide. The results of this experiment are given in Fig. 6. Since the dissociation constant for this flavoprotein is very small, a considerable part of the added flavin is bound to the protein; thus the dissociation constant is not equal to the total flavin concentration when

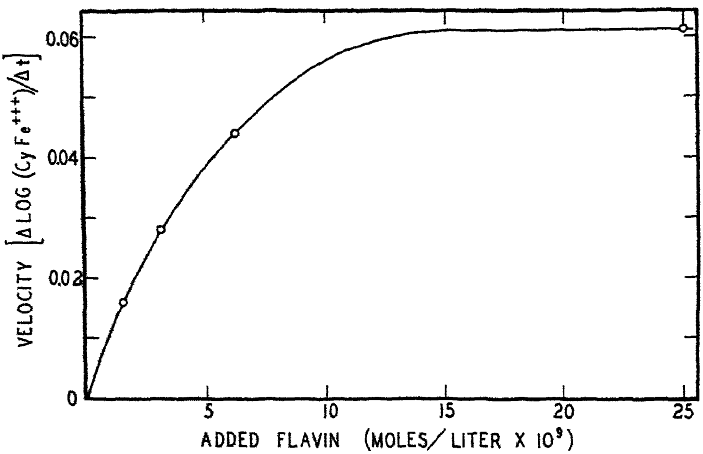

FIG. 6. Rate of reduction of cytochrome c as a function of flavin concentration. Increasing concentrations of flavin$_{c.r.}$ added to 0.92 γ of protein$_{c.r.}$.

the activity is 50 per cent that of maximum. Rather two points on the curve must be used and the constant determined by the solution of simultaneous equations.

The value of the dissociation constant K can be determined from the following considerations. Consider two points on the curve, points (1) and (2). Let V_1 be the rate corresponding to point (1) and V_2 the rate for point (2). $V_{max.}$ is the maximum rate determined with excess flavin. P_t = total protein concentration, P_1 and P_2 = free protein, E_1 and E_2 = enzyme concentration, F_{t1} and F_{t2} = total flavin, and F_1 and F_2 = free flavin at points (1) and (2) respectively.

$$P_t = E_1 + P_1 = E_2 + P_2$$

$$F_{t1} = F_1 + E_1; F_{t2} = F_2 + E_2$$

$$E_1 = P_t \frac{V_1}{V_{max.}}; E_2 = P_t \frac{V_2}{V_{max.}}$$

$$K = \frac{P_1 \times F_1}{E_1} = \frac{P_2 \times F_2}{E_2}$$

It can then be shown that

$$P_t = \frac{\left(\frac{V_{max.} - V_1}{V_1} \times F_{t1}\right) - \left(\frac{V_{max.} - V_2}{V_2} \times F_{t2}\right)}{\frac{V_2 - V_1}{V_{max.}}}$$

With P_t known, the value of K can readily be calculated. The two upper points on the curve were used in this determination, since the experimental errors are less than those for the lowest activity. The value of the constant so determined is 1×10^{-9} mole liter^{-1}. Since the rate of the reaction is limited by the triphosphopyridine nucleotide concentration, it is assumed that most of the flavoprotein is in the oxidized state during the course of the reaction. The dissociation constant is therefore that for the oxidized form.

By the same sort of calculation we have determined the dissociation constants for the "old" and "new" yellow enzymes. These values together with those for the cytochrome reductase and amino acid oxidase are as follows:

Cytochrome *c* reductase..	1×10^{-9} mole $\times$ liter^{-1}
"New" yellow enzyme..........	27×10^{-9} " $\times$ "
"Old" " "	60×10^{-9} " $\times$ "
Amino acid oxidase.............	250×10^{-9} " $\times$ "

The value for the dissociation constant of the amino acid oxidase was determined by Warburg and Christian (18). The cytochrome reductase is considerably more stable with respect to dissociation than are these other flavoproteins.

Prosthetic Group of Cytochrome c Reductase Exchanged with That of "Old" Yellow Enzyme

A preparation of "old" yellow enzyme was made by Mr. H. Persky of this laboratory according to the directions given by

Warburg and Christian (1). The enzyme was further purified by adsorption on aluminum hydroxide gel and subsequent elution with $(NH_4)_2SO_4$, a method first suggested by Kuhn and Sörensen (19).

The prosthetic group of the "old" yellow enzyme, alloxazine mononucleotide, was obtained by following the directions of Theorell (13).

To obtain the protein component, it was necessary to purify the enzyme to a greater extent than for the preparation of the prosthetic group. This was carried out by adsorption with $Ca_3(PO_4)_2$.

4 cc. of the "old" yellow enzyme as previously prepared (3.6×10^{-7} mole) were dialyzed for 2 days. The enzyme was then adsorbed on 250 mg. of $Ca_3(PO_4)_2$ gel. After centrifugation the calcium phosphate with the adsorbed enzyme was washed with 0.05 M phosphate buffer, pH 6.1. The enzyme was then eluted twice with 3 cc. of alkaline $(NH_4)_2SO_4$.

The protein was separated from the prosthetic group by the method of Warburg and Christian (18) which involves the splitting with acid in ammonium sulfate solution.

Prosthetic Group of "Old" Yellow Enzyme Replacing the Prosthetic Group of Cytochrome c Reductase—To determine whether the prosthetic group of cytochrome *c* reductase is alloxazine mononucleotide the prosthetic groups of both the "old" yellow enzyme and of the reductase were added to an excess of the reductase protein. Under these conditions the reaction velocity is a function of the flavin concentration. The activities of the two flavins were compared by the test previously described (Table IV). The results are given in Table VII.

These results show that the activities of the two prosthetic groups are quantitatively the same and that in all likelihood the prosthetic group of cytochrome *c* reductase is alloxazine mononucleotide.

Prosthetic Group of Cytochrome c Reductase Replacing the Prosthetic Group of "Old" Yellow Enzyme—In the following experiment the prosthetic groups of both yellow enzymes are combined with the protein of the "old" yellow enzyme. The activities of these synthesized enzymes were determined manometrically under the conditions described by Theorell (13). The results are apparent in Table VIII.

In Experiments 3 and 4 of Table VIII it is again demonstrated that the activities of the two prosthetic groups are the same within experimental error, and that they are very probably identical. The determination reported in Experiment 5 was made to demonstrate that excess protein was present; that the flavin determines the rate of oxygen uptake.

Prosthetic Group of Cytochrome c Reductase Replacing That of d-Amino Acid Oxidase

The previous experiments leave little doubt that the prosthetic group of cytochrome *c* reductase is alloxazine mononucleotide.

TABLE VII

Activity of Flavins

$Protein_{c.r.}$ = 0.92 γ per cc.

Flavin of cytochrome reductase, 0.31 × 10^{-11} mole per cc.			Flavin of "old" yellow enzyme, 0.31 × 10^{-11} mole per cc.		
t	$8 + \log(CyFe^{+++})$	$\frac{\Delta \log(CyFe^{+++})}{\Delta t}$	t	$8 + \log(CyFe^{+++})$	$\frac{\Delta \log(CyFe^{+++})}{\Delta t}$
min.			*min.*		
0	0.710		0	0.720	
1	0.663	0.047	1	0.675	0.045
2	0.625	0.043	2	0.636	0.042
3	0.583	0.042	3	0.595	0.042
4	0.542	0.042	4	0.551	0.040
5	0.490	0.044	5	0.528	0.041
Average...........		0.044			0.042

Nevertheless we tested it in the following system which is specific for alloxazine-adenine dinucleotide, the prosthetic group of *d*-amino acid oxidase. Alloxazine-adenine dinucleotide was prepared by Mr. B. Block of this laboratory according to the directions given by Warburg and Christian (18). The protein component of the amino acid oxidase was made in accordance with the procedure of Negelein and Brömel (14). The results of these experiments are contained in the Table IX.

In every test we have found the prosthetic group of cytochrome *c* reductase to have the same activity as alloxazine mononucleotide. The results of Table IX show clearly that it is not alloxazine-adenine dinucleotide.

TABLE VIII

Replacement of Prosthetic Group of "Old" Yellow Enzyme by That of Cytochrome Reductase

Protein = protein of "old" yellow enzyme; 38°; cup, 0.1 cc. of 2 N KOH; gas space, oxygen.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
	2.6 cc. water 18 mg. hexose monophosphate (K salt) 2 mg. *Zwischenferment* 0.52 mg. KCN 0.08 " triphosphopyridine nucleotide	→	→	→	→
	1.0 mg. protein	2×10^{-9} mole flavin$_{c.r.}$	1.0 mg. protein 2×10^{-9} mole flavin$_{c.r.}$	1 mg. protein 2×10^{-9} mole alloxazine mononucleotide	1 mg. protein 4×10^{-9} mole alloxazine mononucleotide
C.mm. O_2 taken up in 10 min.	2	1.5	38	42	74

TABLE IX

Prosthetic Group of Cytochrome c Reductase Replacing That of d-Amino Acid Oxidase

Protein = protein of *d*-amino acid oxidase; 38°; cup, 0.1 cc. of 2 N KOH; gas phase, oxygen.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
	2.6 cc. 0.04 M pyrophosphate buffer, pH 8.1 0.47 mg. protein 9.0 mg. *dl*-alanine	→ 1.5×10^{-9} mole flavin$_{c.r.}$	→ 1.5×10^{-9} mole alloxazine mononucleotide	→ 1.5×10^{-9} mole alloxazine-adenine dinucleotide	→ 3.0×10^{-9} mole alloxazine-adenine dinucleotide
C.mm.O_2 taken up in 10 min.	6	20	19	122	210

Determination of Phosphorus Content of Prosthetic Group

To confirm the conclusion that the prosthetic group is alloxazine mononucleotide, we determined the number of phosphorus atoms per molecule of $\text{flavin}_{\text{c.r.}}$. In alloxazine mononucleotide this number is 1 and in the dinucleotide it is 2.

The phosphorus determination was carried out according to the following procedure. To 2.0 cc. of the solution containing 0.668×10^{-7} mole of enzyme, 0.1 cc. of 40 per cent trichloroacetic acid was added. Under these conditions the enzyme is split and the protein precipitated. After centrifugation the protein was washed with 2 cc. of 2 per cent trichloroacetic acid and discarded. The supernatant and the wash solution which contain the prosthetic group were combined and evaporated to dryness. The residue was digested with 1.0 cc. of 10 N H_2SO_4 and a drop of perhydrol. Water, molybdate, and sulfonic acid reagent were added in accordance with the procedure given in the following paper (20). The blue phosphomolybdate complex was developed by heating to 100° and from the light absorption at 670 mμ ($I_0/I = 2.47$) in a 1.98 cm. cell the amount of phosphorus was calculated to be 2.5 γ.

The 2.5 γ of phosphorus correspond to 0.80×10^{-7} mole of phosphorus. The proportion of phosphorus to flavin = 0.80/0.668 = 1.2. In view of the fact that the enzyme had been treated with phosphate during preparation, it is understandable that this ratio could be somewhat high, but this result together with all the foregoing experiments indicates that the prosthetic group contains 1 phosphate atom per molecule and is alloxazine mononucleotide.

DISCUSSION

The very high activity of cytochrome *c* reductase both in oxidizing reduced triphosphopyridine nucleotide and in reducing oxidized cytochrome *c* makes it seem highly probable that this enzyme constitutes a missing link in the oxidation-reduction chain. Although the potential of cytochrome *b* lies between that of cytochrome *c* and the flavoproteins, there is now no necessity to include it in this part of the oxidation mechanism. The enzymatic functions of both cytochromes *a* and *b* are therefore still left to be elucidated.

The fact that the prosthetic group of cytochrome *c* reductase is alloxazine mononucleotide places this coenzyme in a more significant position as a biologically important compound. The only

other known flavoprotein having this same prosthetic group is the "old" yellow enzyme.

In view of the unstable nature of cytochrome *c* reductase it is now easy to understand the reason that this substance previously escaped detection. If cytochrome *c* reductase is subjected to the same treatment as that used in the isolation of other flavoproteins, its activity toward cytochrome *c* is destroyed. In the preparation of the "old" yellow enzyme Warburg and Christian kept their product in 33 per cent acetone for 24 hours Under these conditions cytochrome *c* reductase loses its activity completely in less than 1 hour. Another of their purification steps involves shaking for 24 hours at 38°. The activity of our enzyme in reducing cytochrome *c* is diminished to the extent of 37 per cent when kept for 10 minutes at 40°. Since we have not yet studied the reaction between the reductase and molecular oxygen, we are not in a position to make any statement regarding the stability of our enzyme in its reaction with oxygen. Inasmuch as the free flavin is autoxidizable, it is conceivable that the activity of our enzyme toward cytochrome *c* may be destroyed while that toward oxygen may not.

SUMMARY

1. A new flavoprotein, cytochrome *c* reductase, which completes the oxidation-reduction chain between hexose monophosphate and cytochrome *c* is reported.
2. A method for isolating this enzyme from yeast is described.
3. The molecular weight of the enzyme was determined to be about 75,000. The purity of the final product was found to be about 87 per cent.
4. The absorption spectrum and the method of titrating cytochrome *c* reductase by cytochrome *c* are described.
5. The analytical method, based upon spectrophotometric rate determinations, is given, together with a somewhat general treatment of the kinetics involved in the test. Minimum values of the rate constants are determined and it is demonstrated that cytochrome *c* reductase reacts specifically with cytochrome *c*, whereas the "old" or "new" yellow enzymes do not.
6. The enzyme can be split into a protein and a prosthetic group which is alloxazine mononucleotide. By recombining these the enzyme can be resynthesized.

7. The evidence that the prosthetic group is alloxazine mononucleotide is given by the interchange of the prosthetic groups of cytochrome c reductase, the "old" yellow enzyme, and the amino acid oxidase. This is supported by a determination of the phosphorus in the prosthetic group.

8. The constant for the dissociation of the oxidized form of the enzyme into its protein and alloxazine mononucleotide is determined to be 1×10^{-9} mole liter^{-1}. This enzyme is considerably less dissociated than any of the other yellow enzymes.

9. The enzyme is very unstable with respect to low pH and to denaturation by heat.

10. A method is given for determining very small amounts (10^{-3} γ) of alloxazine mononucleotide.

We are particularly indebted to the Rockefeller Foundation, without whose help this work could not have been carried out by us. We also wish to acknowledge the help received from Drewry's, Ltd., Anheuser-Busch, Inc., the Canadian Breweries, Ltd., and the Works Progress Administration.

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NOTE ON THE DETERMINATION OF MICROQUANTITIES OF ORGANIC PHOSPHORUS

By B. L. HORECKER, T. S. MA, and ERWIN HAAS

(From the George Herbert Jones Chemical Laboratory of the University of Chicago, Chicago)

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During the course of studies on enzymes in this laboratory (1), it became desirable to have a method of determining quantitatively small amounts (about 1 γ) of phosphorus in protein. The available methods were unsatisfactory either because they demanded a larger sample than could be prepared conveniently or because the excess sulfuric acid necessary for digestion interfered with the determination. Kuttner and Cohen (2) have reported a method which allows the determination of as little as 2.5 γ of phosphorus, but the amount of sulfuric acid which is permitted during the phosphorus determination is insufficient for the preceding digestion. Berenblum and Chain (3) were able to determine less than 1 γ, but their method has the disadvantage that it involves an extraction with small volumes of isobutyl alcohol.

By modifying the method of Fiske and Subbarow (4) and by using the photoelectric spectrophotometer described by Hogness, Zscheile, and Sidwell (5), 1 γ of phosphorus can be determined with an accuracy of ± 3 per cent. In order to insure complete digestion and to avoid any loss of phosphorus, a larger quantity of sulfuric acid is used. The final concentration of sulfuric acid is 2 N instead of 0.5 N as specified in the original method. The blue color of the reduced phosphomolybdic acid complex is developed by heating, as recommended by Benedict and Theis (6). At room temperature the color is stable for several hours. The color intensity is determined spectrophotometrically and the amount of phosphorus in the sample read from the standard determination.

EXPERIMENTAL

Reagents—The following reagents are prepared according to the directions of Fiske and Subbarow.

1. 2.5 per cent ammonium molybdate.
2. 0.25 per cent 1, 2, 4-aminonaphtholsulfonic acid in 15 per cent sodium bisulfite and 0.5 per cent sodium sulfite.

Determination of Unknown Sample—A sample containing 1 to 5 γ of phosphorus (corresponding to 2 to 10 mg. of dry weight in the case of enzyme preparations) is digested with 1 cc. of 10 N sulfuric acid and a drop of perhydrol. The resulting colorless solution is diluted and 0.5 cc. of molybdate solution and 0.2 cc. of naphtholsulfonic acid reagent are added. The final volume is then brought to 5 cc. The solution is heated for 20 minutes in boiling water, cooled, and the light absorption at 6700 Å. determined in an absorption cell having a length of 2.0 cm.

Determination of Standard Phosphorus—A blank solution and standard solutions containing 2, 4, and 6 γ of phosphorus are treated with H_2SO_4 and perhydrol. After formation of the phosphomolybdic acid complex the light absorption is determined as above. The values of log I_0/I are plotted against the amounts of phosphorus. A straight line is obtained. The standard solutions need be determined only once and the phosphorus content of an unknown sample can be read directly from the graph.

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CYTOCHROME C PEROXIDASE

By A. M. ALTSCHUL, RICHARD ABRAMS, and T. R. HOGNESS

(*From the George Herbert Jones Chemical Laboratory of the University of Chicago, Chicago*)

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As the result of a study of the mechanism of the oxidation of reduced cytochrome *c* it was found that cytochrome *c* was readily oxidized by very small concentrations of hydrogen peroxide through the intermediation of an extremely active peroxidase. Further investigation resulted in the isolation and purification of a heretofore unreported enzyme, cytochrome *c* peroxidase, which is specific toward reduced cytochrome *c*. The source of the peroxidase used in this investigation was bakers' yeast.

Peroxidase preparations have been investigated in the past, but it has not been demonstrated that these react with any of the important components of the respiratory system. The test for the activity of a peroxidase has usually been that of determining its ability to catalyze the reaction between hydrogen peroxide and pyrogallol. The enzyme, cytochrome *c* peroxidase, is inactive toward pyrogallol, while conversely peroxidase prepared from horseradish root, is not active toward cytochrome *c*.

In this report we shall describe the chemical properties of cytochrome *c* peroxidase, its constitution, as far as we know it, and the method of its isolation and purification. Due to the unsuspected contamination of reduced cytochrome *c* with hydrogen peroxide produced in the preliminary reduction, this enzyme was first thought to be a soluble cytochrome oxidase and was erroneously reported as such in previous communications (1, 2).

Analytical Test

The over-all reaction for the oxidation of reduced cytochrome *c* by hydrogen peroxide is expressed by the stoichiometric equation,

$$2CyFe^{++} + H_2O_2 + 2H^+ = 2CyFe^{+++} + 2H_2O \qquad (1)$$

In this equation reduced cytochrome c is denoted by $CyFe^{++}$ and the oxidized form by $CyFe^{+++}$. The rate of oxidation of the cytochrome, besides being dependent upon the cytochrome c and hydrogen peroxide concentrations, is a function of the concentration of the enzyme, cytochrome c peroxidase. This fact is the basis of the test for measuring the cytochrome peroxidase activity.

As shall be shown experimentally, the rate of oxidation of reduced cytochrome c is directly proportional to the concentrations of cytochrome c and the peroxidase. This proportionality is expressed in the following rate equation,

$$-\frac{d(\mathrm{CyFe^{++}})}{dt} = K(\mathrm{CyFe^{++}})(E)(\mathrm{H_2O_2})^x \tag{2}$$

in which K is the rate constant and (E) the concentration of the peroxidase. Under the conditions prevailing in the test the rate of the reaction is practically independent of the concentration of the hydrogen peroxide which is present in large excess. Therefore the equation may be written,

$$-\frac{d(\mathrm{CyFe^{++}})}{dt} = K'(\mathrm{CyFe^{++}})(E) \tag{3}$$

or

$$-\frac{d\log_{10}(\mathrm{CyFe^{++}})}{dt} = \frac{K'}{2.3}(E) \tag{4}$$

In any one experiment the peroxidase concentration (E) does not vary and $d \log (\mathrm{CyFe^{++}})/dt$ therefore remains constant during the course of the reaction. A plot of $\log (\mathrm{CyFe^{++}})$ against time, t, should give a straight line. In Fig. 1 the results of a typical experiment show that this is the case. The activity of the enzyme is determined by the slope of the line in such a plot. A plot of the slopes of such straight lines obtained for different peroxidase concentrations against the enzyme concentration (E) should also give a straight line. This relationship is shown in Fig. 2 in which slopes or values of $d \log (\mathrm{CyFe^{++}})/dt$ obtained for different enzyme concentrations appear as functions of the added enzyme. The rate of the reaction may also be a function of the H^+ ion concen-

tration, but since all tests are made at a constant buffered pH, the dependence on pH is not involved.

A cytochrome peroxidase unit of activity is defined as that amount of peroxidase which gives a value of 1 min.$^{-1}$ for $-d \log (\text{CyFe}^{++})/dt$. The relative purity of any preparation is determined by the number of enzyme units per mg. of protein and is denoted by the symbol, Q.

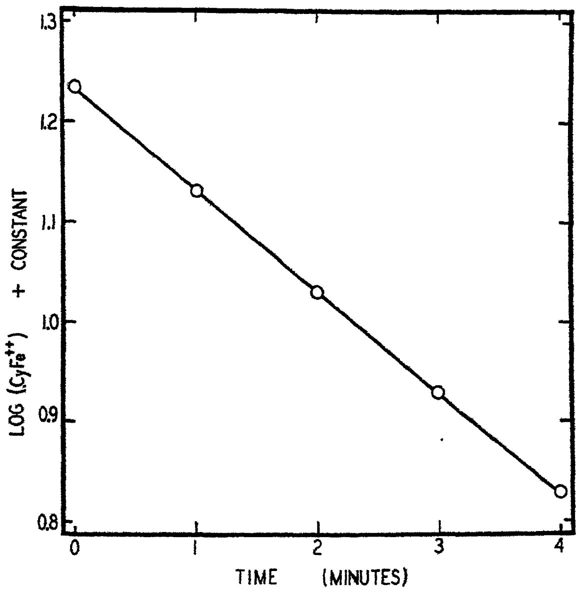

FIG. 1. A typical plot showing the method used to determine the rate of oxidation of reduced cytochrome c by hydrogen peroxide as catalyzed by cytochrome c peroxidase.

A spectrophotometric method was used to determine the concentration of reduced cytochrome c. Monochromatic light, wavelength 5500 Å., corresponding to the peak of the α band of reduced cytochrome c was used for analysis. For a mixture of oxidized and reduced cytochrome c the relationship between light absorption and concentration is expressed by the equation,

$$\text{Log}\left(\frac{I_0}{I}\right) = \alpha_O(\text{CyFe}^{+++})l + \alpha_R(\text{CyFe}^{++})l \tag{5}$$

in which α_O and α_R are the extinction coefficients of oxidized and reduced cytochrome respectively; l is the length of the absorption cell. After all the cytochrome has been oxidized,

$$\text{Log}\left(\frac{I_0}{I}\right)_\infty = \alpha_O(\text{Cy}_t)l \tag{6}$$

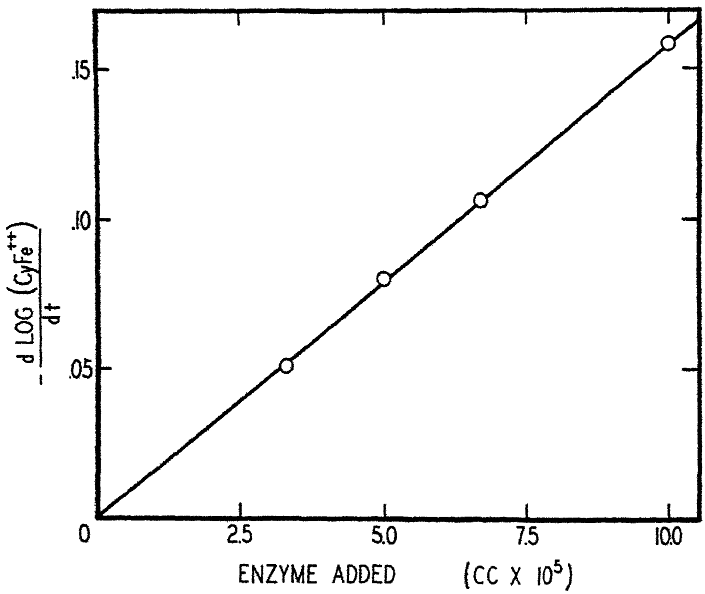

Fig. 2. The rate of oxidation of reduced cytochrome *c* by hydrogen peroxide as a function of the concentration of cytochrome *c* peroxidase.

(Cy_t) is the total concentration of cytochrome. When Equations 5 and 6 are combined, the concentration of reduced cytochrome at any time is given by Equation 7.

$$(\text{CyFe}^{++}) = \frac{\log\left(\frac{I_0}{I}\right) - \log\left(\frac{I_0}{I}\right)_\infty}{(\alpha_R - \alpha_O)l} \tag{7}$$

The cytochrome *c* used in the tests was prepared from horse heart according to the method of Keilin and Hartree (3). After dialysis, the cytochrome *c* was precipitated with 4 volumes of

cold acetone and dried *in vacuo*. Stock solutions for the test were made by dissolving 100 mg. of cytochrome *c* in 5 cc. of 0.01 N HCl.

The following is the procedure used in carrying out the test illustrated in Fig. 1. Cytochrome *c* from the stock solution is diluted with 0.02 M phosphate buffer, of pH 7.4, to a concentration of 1.5×10^{-8} mole per cc. For reproducible results, it is important that the buffer concentration be low and that the same total cytochrome *c* concentration be used for all tests. The diluted cytochrome *c* solution is reduced with hydrogen and 10 per cent palladium asbestos and then filtered. It is not necessary to add hydrogen peroxide to the test solution, because enough is formed from the dissolved oxygen during the reduction (4). The concentration of hydrogen peroxide formed in this reduction is 4.5×10^{-8} mole per cc.—a 6-fold excess over that of the cytochrome *c*.

To 3 cc. of the filtered solution in a 1 cm. absorption cell is added 0.1 cc. of enzyme solution containing enough peroxidase to cause complete oxidation of the reduced cytochrome *c* in about 5 minutes. Log I_0/I is then measured with the spectrophotometer (5) at appropriate intervals of time. Finally, to obtain conveniently the value of log $(I_0/I)_\infty$, 0.05 cc. of 10^{-3} M potassium ferricyanide solution is added to oxidize the cytochrome *c* completely. The values of log($CyFe^{++}$) are then calculated and plotted against t, and a straight line is obtained. From the slope of this line the concentration of the enzyme in the particular preparation is determined. Reduced cytochrome *c* is oxidized very slowly in the absence of the enzyme. This rate should be determined in a control experiment and the appropriate corrections made.

Isolation and Purification

Starting Material—Bakers' yeast affords a rich source of cytochrome *c* peroxidase. Anheuser-Busch's bakers' yeast, after being washed, pressed, and dried overnight on copper screens, is finely ground in a coffee-mill. Since dry yeast slowly loses its activity, the yeast should always be dried a short time before using.

The following procedure is based upon the results of a large number of preparations. The numerical figures for purity and yield represent average values.

Step 1. Extraction by Autolysis—5 kilos of dried yeast are

added to 15 liters of distilled water and the mixture is allowed to digest at 25° for 24 hours. (The extraction time varies somewhat for different batches of yeast, and it is advisable to determine the optimum time by means of a small test run before beginning the large preparation.) After 24 hours, the suspension is cooled and centrifuged. 6 liters of cloudy supernatant liquid (Solution A) are obtained. This may be kept at 0° for at least 2 days without any loss of activity. Activity 400,000 enzyme units; $Q = 1$.

Step 2. Ammonium Sulfate-Trichloroacetic Acid Precipitation—Hereafter, all operations are carried out at 0°. To 6 liters of Solution A are added 2.1 kilos of ammonium sulfate (350 gm. per liter) and 330 cc. of 20 per cent trichloroacetic acid (55 cc. per liter). A heavy precipitate forms. After standing at 0° for ½ hour, the mixture is centrifuged and the supernatant liquid is discarded. The precipitate is extracted twice with a total volume of 1.2 liters of water. After centrifugation, the extracts which contain the enzyme are combined, the pH adjusted to 3.8, and then the solution is dialyzed for 12 to 15 hours against cold, running, distilled water. The cloudy dialysate is clarified by centrifugation, yielding 1620 cc. of clear brown solution (Solution B). 180,000 enzyme units; $Q = 15$; purification 15-fold; yield 45 per cent.

Step 3. Alcohol Fractionation—After Solution B has been diluted until the dry weight is 5 mg. per cc., the pH is adjusted to 4.2. To 3240 cc. of this solution are added 810 cc. of ice-cold 95 per cent ethyl alcohol, the total alcohol concentration then being 19 per cent by volume. After standing at 0° for 15 minutes, the precipitate is separated by centrifugation and discarded. To the clear supernatant are added 1350 cc. more of cold alcohol to bring the concentration of the latter up to 38 per cent. Again the mixture is allowed to stand at 0° for 15 minutes and then it is centrifuged. The resulting red-brown precipitate is dried *in vacuo* in the refrigerator. The dry powder so obtained may be stored in a desiccator at 0° for several months without loss of activity. (It has proved convenient to stop at this point in the preparation and store up large quantities of the dry powder before going ahead with the purification.) 2.5 gm. of dry powder are extracted with 125 cc. of cold distilled water. After the mixture is centrifuged and the insoluble residue discarded, 120 cc. of a red, cloudy solu-

tion (Solution C) are obtained. 72,000 enzyme units; $Q = 75$; purification 5-fold; yield 40 per cent.

Step 4. Adsorption and Elution—To 120 cc. of Solution C are added 24 cc. of γ aluminum hydroxide (6). (The aluminum hydroxide has a dry weight of 7.6 mg. per cc.) After standing for 5 minutes, the mixture is centrifuged, and the supernatant liquid discarded. The precipitate is washed three times, each time with 120 cc. of water; the wash water is discarded. The enzyme is then eluted from the aluminum hydroxide with successive lots of 3 cc. of 18 per cent saturated ammonium sulfate until the last eluate appears perfectly colorless. Five elutions generally suffice. The brown-colored eluates are combined and dialyzed for 38 hours against cold, running, distilled water. A precipitate which forms during dialysis is removed by centrifugation, leaving 20 cc. of clear brown solution (Solution D). This solution is very stable and may be kept for several weeks at 0°. 25,000 enzyme units; $Q = 450$; purification 6-fold; yield 35 per cent.

Step 5. Alcohol Precipitation—To 20 cc. of Solution D, 0.2 cc. of 0.1 M acetate buffer of pH 4.63 is added. The resulting pH is about 4.7. Then 7 cc. of alcohol are added at 0° to bring the alcohol concentration to 25 per cent. After standing at 0° for ½ hour the suspension is centrifuged, and the brown precipitate dried *in vacuo* in the refrigerator. The dry powder is extracted with 4 cc. of cold water. After centrifugation the residue is discarded and a clear, dark brown solution (Solution E) is obtained. 12,500 enzyme units; $Q = 800$; purification 1.8-fold; yield 50 per cent.

The over-all purification of 800-fold is obtained with a 3.2 per cent yield. The degree of purification is based upon the concentration of the enzyme in the original extract and not upon the whole yeast.

Absorption Spectrum

The purest preparation obtained had a brown color and gave the spectrum shown in Fig. 3. Absorption peaks were found at 2700 and at 4100 Å. Upon reduction with sodium hydrosulfite, the peak at 4100 Å. disappeared and new peaks appeared at 4375 and 5600 Å. The absorption in the ultraviolet region is

typical for proteins; that in the visible region, for hemin compounds. This spectrum is similar to that of peroxidase obtained from horseradish (7).

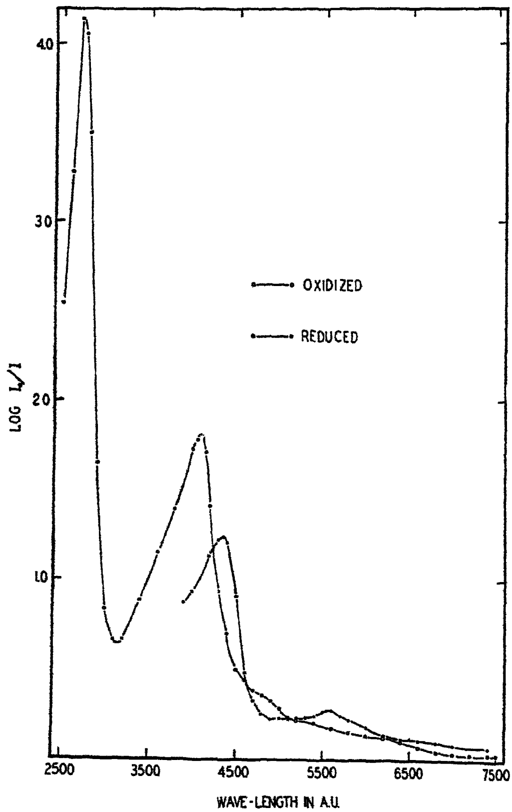

FIG. 3. Spectra of oxidized and reduced cytochrome *c* peroxidase preparation.

Cytochrome c-Hydrogen Peroxide Reaction

In order to demonstrate conclusively the function of the enzyme, it was necessary to obtain reduced cytochrome *c* free of both hydrogen peroxide and any substrate which might react rapidly with the hydrogen peroxide or which might otherwise inhibit the enzyme. The method of reduction which was found to be most satisfactory

was the enzymatic procedure described by Haas, Horecker, and Hogness (8). By the proper adjustment of the concentrations of the reactants (hexose monophosphate, *Zwischenferment*, triphosphopyridine nucleotide, and cytochrome reductase), the rate of reduction of cytochrome *c* can be made as slow as desired. In the following experiments, the cytochrome *c* was completely reduced in about 12 hours.

Cytochrome *c* so reduced exhibits no measurable rate of autoxidation. The addition of a large amount of cytochrome *c* peroxidase to this solution of reduced cytochrome *c* causes no oxidation. Since the cytochrome *c* solution is saturated with air, oxygen obviously is not an oxidant with this enzyme. If the reduced cytochrome *c* is mixed with an excess (10- to 20-fold) of hydrogen peroxide, it is very slowly autoxidized. Upon addition of both hydrogen peroxide and enzyme, however, the rate of oxidation is very rapid. This is illustrated by the following experiment. 3 cc. of reduced cytochrome *c* (concentration $= 1.4 \times 10^{-8}$ mole per cc.) were mixed with 0.1 cc. of solution containing 26.8×10^{-8} mole of hydrogen peroxide. This was a 12.5-fold excess of the latter. The rate of oxidation of the reduced cytochrome was measured in the spectrophotometer. 6 minutes after the first reading, 0.1 cc. of an enzyme preparation (0.088 enzyme unit) was added. The increase in rate as shown in Fig. 4 demonstrates the catalytic action of the enzyme.

In the foregoing case, a small amount of enzyme was used in order to obtain a measurable rate. If larger amounts (1 or more enzyme units) are added, the oxidation of cytochrome *c* is instantaneous. With such large concentrations of enzyme, it is possible to titrate reduced cytochrome *c* with hydrogen peroxide.

The titration of the reduced form of cytochrome *c* was carried out as follows: 3 cc. of a reduced cytochrome *c* solution (2.95×10^{-8} mole per cc.) which was about 30 per cent reduced was placed in a 1 cm. absorption cell. To this was added 0.1 cc. (110 enzyme units) of a peroxidase preparation of $Q = 710$. This caused no oxidation of the cytochrome *c*. Hydrogen peroxide was added in small amounts, 0.335×10^{-8} mole at a time, and log I_0/I determined immediately after each addition. When all the cytochrome *c* was oxidized, further addition of hydrogen peroxide produced no change in the absorption. Log I_0/I was corrected

for dilution, and the total amount of reduced cytochrome c remaining after each addition of hydrogen peroxide was then calculated.

The solid line in Fig. 5 is the theoretical curve calculated with the assumption that the reaction between reduced cytochrome c and hydrogen peroxide proceeds according to Equation 1. The experimentally determined points coincide with this line. Obviously, this reaction can be used in the determination of small amounts of hydrogen peroxide and was so used to determine the

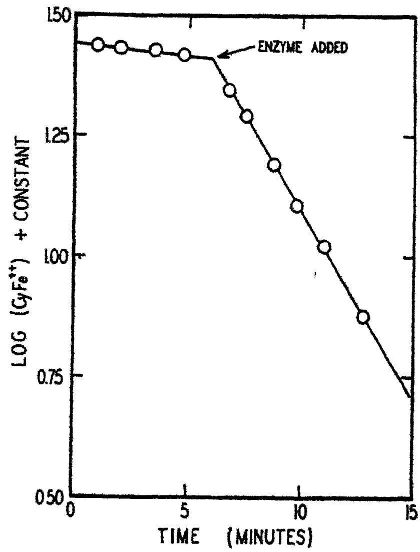

FIG. 4. Experiment demonstrating the activity of cytochrome c peroxidase.

amount of hydrogen peroxide formed in the catalytic reduction of cytochrome c by hydrogen and palladium.

The above experiments demonstrate the enzymatic function of cytochrome c peroxidase. It catalyzes the reaction between reduced cytochrome c and hydrogen peroxide.

Comparison of Peroxidases

Since Willstätter's early work on peroxidase, the standard test for peroxidase activity has been a determination of its activity as a catalyst for the oxidation of pyrogallol to purpurogallin by

hydrogen peroxide. Cytochrome peroxidase, however, does not catalyze this reaction. To determine whether peroxidase as obtained from horseradish root has any catalytic activity upon the oxidation of cytochrome *c*, a preparation of this enzyme was made by the method described by Elliott (9). That this peroxidase is inactive toward cytochrome *c* is shown by the following results

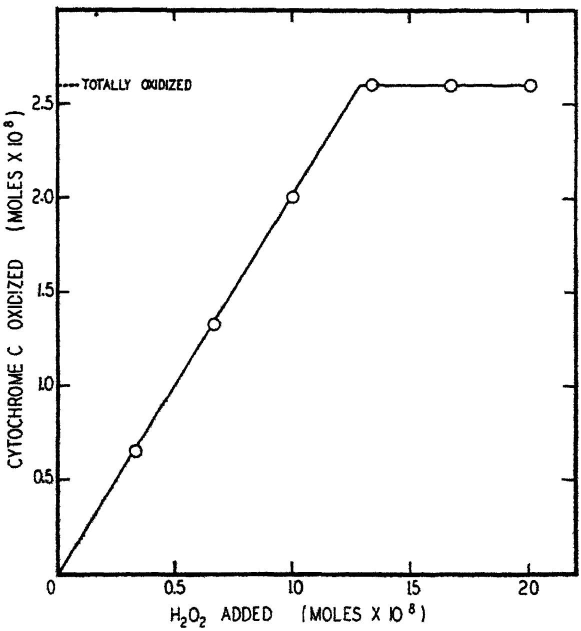

FIG. 5. Titration of the reduced form of cytochrome *c* by hydrogen peroxide. The solid line is the theoretical curve calculated with the assumption that the reaction between reduced cytochrome *c* and hydrogen peroxide proceeds according to Equation 1. The points were determined experimentally.

which summarize the experiments comparing the activities of horseradish and cytochrome *c* peroxidases: cytochrome *c* peroxidase, *PZ* 0.9, *Q* 800; horseradish peroxidase, *PZ* 130, *Q* 4.3. (*PZ*, the purpurogallin number, is defined as the number of mg. of purpurogallin formed in 5 minutes at 20° by 1 mg. of peroxidase preparation, from 12.5 mg. of hydrogen peroxide and 1.25 gm. of pyrogallol (10).) The very small activity of cytochrome *c*

peroxidase toward pyrogallol and that of horseradish peroxidase toward cytochrome *c* are negligible. Thus it is clear that these two are distinctly different enzymes.

Properties of Cytochrome c Peroxidase

Heat Lability—Both the original extract and the purified solution have about the same sensitivity to heat. 85 per cent of the activity of cytochrome *c* peroxidase is destroyed if the enzyme is kept at 55° for 5 minutes.

Cyanide Inhibition—The enzyme is extremely sensitive to small concentrations of potassium cyanide; it is completely inhibited by 3×10^{-4} M KCN. Both the original extract and the purified preparation display the same sensitivity to cyanide.

Salt Effect—The activity of this enzyme is affected markedly by the salt concentration of the test medium. An increase in the buffer strength of the test solution from 0.02 to 0.10 M phosphate results in an 87 per cent inhibition of the activity. This inhibition seems to be a general salt inhibition and not specific for phosphate ions, inasmuch as 0.10 M NaCl concentration inhibits the activity to the extent of 80 per cent. This effect recalls a similar result obtained in the study of the oxygen uptake of purified hemoglobin solution (11). Similar salt effects have been found for both catalase and horseradish peroxidase (12).

Catalase Inhibition—As might be expected, catalase when present in relatively large amounts prevents the oxidation of reduced cytochrome *c*. If the catalase is present at a concentration which is of the same order of magnitude as that of the peroxidase, the rate of the reaction continually decreases with time because of the destruction of the hydrogen peroxide by the catalase.

Chemical Constitution

Hemin Content—The foregoing facts, that the spectrum of the cytochrome *c* peroxidase preparation has a hemin-like structure and that cyanide inhibits its activity, pointed to the likelihood that cytochrome *c* peroxidase is a hemin-protein compound. This conclusion was confirmed by experiments in which a reduced pyridine hemochromogen was produced from the enzyme when it was treated with pyridine and a reducing agent. The spectrum of the hemochromogen produced in this way was identical with that obtained from blood hemin.

In determining the hemin content a modification of the method of Kuhn, Hand, and Florkin (7) was used. To 0.5 cc. of a solution of the enzyme in water, 0.03 cc. of 5 M NaOH, 0.1 cc. of pyridine, and 0.2 cc. of a neutralized 0.1 M solution of ascorbic acid were added. The formation of the pink hemochromogen was complete within 5 minutes.

Reduced pyridine hemochromogen has absorption peaks at 5275 and 5575 Å. with a minimum at 5400 Å. The test solution was placed in a 1 cm. absorption cell and the extinction measured at 5400 and 5575 Å. by means of the photoelectric spectrophotometer. From these absorption data the concentration of hemin was calculated from the formula,

$$(\text{Hemin}) = \frac{\log\left(\frac{I_0}{I}\right)_{5575} - \log\left(\frac{I_0}{I}\right)_{5400}}{(\alpha_{5575} - \alpha_{5400})} \tag{8}$$

Measurements on a standard solution gave $\alpha_{5575} - \alpha_{5400} = 27.3$ cm.^{2} per mg. hemin. The concentration of hemin is expressed in mg. per cc. With this method it is possible accurately to determine 1 γ of hemin. The use of ascorbic acid rather than sodium hydrosulfite as the reducing agent is desirable, inasmuch as the latter often causes a progressively increasing turbidity which interferes with the optical measurements. Also, by the use of the difference in absorption between 5575 and 5400 Å. rather than the absolute values, errors due to absorbing impurities and scattering of light are minimized.

Sample 1—A preparation having a value of $Q = 806$ and a dry weight of 1.81 mg. per cc. was analyzed for hemin by the above procedure. It contained 0.00542 mg. of hemin per cc. The per cent hemin was 0.30.

Sample 2—A preparation with a value of $Q = 705$ and a dry weight of 2.653 mg. per cc. contained 0.00804 mg. of hemin per cc. The per cent hemin was 0.30.

Thus two different samples of the peroxidase with not very greatly different activities showed the same hemin content. The hemin content of this sample of cytochrome peroxidase may be compared with the hemin content of both catalase and hemoglobin. The hemin content of catalase is 0.92 per cent, while that of hemoglobin is 3.4 per cent. This indicates that if the molecular weight of cytochrome *c* peroxidase is the same as that of catalase the

purest preparations are about 30 per cent pure. On the other hand if the hemin to protein ratio is the same as that for hemoglobin a product of only 10 per cent purity has been obtained.

Copper Content—A qualitative spectrographic analysis showed that, in addition to iron, significant amounts of copper were present in the purified enzyme preparations. A quantitative determination of copper was therefore made. The method used was that of Eisler, Rosdahl, and Theorell (13). Doubly distilled water was used in making up the reagents. A few drops of 30 per cent H_2O_2 were used instead of perchlorate to remove all color. The solutions of enzyme which were analyzed were Solution E preparations, obtained in step (5) of the purification, for which double distilled water was used to dissolve the dried alcohol precipitate.

Sample 1—1 cc. of Solution E containing 2.65 mg. of protein and having a value of 705 for Q was found to contain 2.85 γ of Cu; per cent Cu = 0.11.

Sample 2—1 cc. of Solution E containing 1.81 mg. of protein (Q = 806) was found to contain 1.56 γ of Cu; per cent Cu = 0.086.

Upon purification the copper content decreased slightly. This, however, is not in itself sufficient evidence to show that the enzyme does not contain copper.

The sodium salt of diethyl dithiocarbamate is a specific inhibitor for copper catalysts. Kubowitz (14) has reported the complete inhibition of polyphenol oxidase by this reagent. Stotz, Harrer, and King (15) found that with a 100-fold excess of inhibitor, the inhibition of copper-containing oxidases was 70 to 100 per cent. With a 150,000-fold excess of diethyl dithiocarbamate no inhibition of cytochrome *c* peroxidase activity was exhibited. It therefore seems highly probable that the copper found in the cytochrome *c* peroxidase preparations bears no relationship to the catalytic activity of the enzyme.

DISCUSSION

From the data already at hand it is possible to make a comparison of the rate of destruction of H_2O_2 by catalase with the rate of H_2O_2 utilization by the reaction involving cytochrome *c* peroxidase.

Under the conditions used in the test for peroxidase the initial

concentration of cytochrome c is 1.5×10^{-8} mole per cc., the same order of magnitude as its concentration in skeletal tissue. The hydrogen peroxide concentration is 5×10^{-8} mole per cc. With this concentration of reactants and with a peroxidase preparation for which $Q = 800$ the rate at which hydrogen peroxide reacts is equal to 5×10^{-5} mole per minute per mg. of enzyme.

The destruction of H_2O_2 by catalase takes place in accordance with the rate equation

$$-\frac{dH_2O_2}{dt} = k(H_2O_2)$$

For crystalline beef catalase $k = 30$ and for horse catalase $k = 63$. With an H_2O_2 concentration of 5×10^{-8} mole per cc., as in the peroxidase test, the rate of destruction of the H_2O_2 is 7.5×10^{-5} mole per minute per mg. of beef catalase, and 15.8×10^{-5} mole per minute per mg. of horse catalase. In the above the activity of pure catalase has been compared with that of an impure sample of the peroxidase. A better comparison might be one involving the activity per mg. of hemin in the two enzymes. Pure catalase contains 0.92 per cent hemin, whereas the purest preparation of cytochrome peroxidase contains 0.30 per cent. The activities both on the basis of the hemin content and on the basis of dry weight are given in Table I. On the basis of hemin content cytochrome c peroxidase has about the same activity as that of horse catalase and a greater activity than that of beef catalase.

TABLE I

Rates of Utilization of Hydrogen Peroxide

	H_2O_2 per mg. enzyme per min.	H_2O_2 per mg. hemin per min.
	mole	*mole*
Cytochrome c peroxidase	5.0×10^{-5}	16.7×10^{-3}
Beef catalase	7.5×10^{-5}	8.16×10^{-3}
Horse catalase	15.8×10^{-5}	17.2×10^{-3}

Dry bakers' yeast contains about 7×10^{-10} gm. of iron as catalase per gm. of yeast (16), while 1 gm. of dry bakers' yeast contains about 18×10^{-8} gm. of peroxidase iron. In yeast there-

fore there is about a 250-fold excess of peroxidase over that of catalase. The determination of the peroxidase content of tissue will be somewhat difficult, inasmuch as both catalase and cytochrome oxidase interfere with the peroxidase test.

The fact that the absorption spectrum of the cytochrome *c* peroxidase preparation is very much like that of horseradish peroxidase leads to the assumption that both of these enzymes have a similar structure. The proteins are not alike inasmuch as cytochrome *c* peroxidase is specific toward cytochrome *c* and is not active toward pyrogallol. The question may arise as to whether or not these two enzymes are identical; their different activities may be due to a combination of inhibitors and accelerators. The difference between the activity of these two enzymes could conceivably be explained by the assumption that the cytochrome *c* peroxidase consists of two enzymes which are necessary for the oxidation of cytochrome *c* together with an inhibitor which prevents the reaction with pyrogallol. This, however, seems very unlikely. We are therefore led to the conclusion that cytochrome *c* peroxidase and horseradish peroxidase are two different entities.

Whether or not this enzyme is important in all respiratory systems involving cytochrome *c* is at the moment not easy to ascertain. In fact, very little is known about the importance of hydrogen peroxide itself in cellular respiration; nor has it ever been directly demonstrated that hydrogen peroxide is present in intact animal tissue. The implicit assumption has been made that any hydrogen peroxide produced in the respiratory process is destroyed by catalase. However, some respiratory systems do not contain large concentrations of catalase and, in such systems at least, cytochrome *c* peroxidase may play a significant rôle. Even in systems rich in catalase it is conceivable that the cytochrome *c* peroxidase, because of its very great activity, may successfully compete with catalase for the hydrogen peroxide.

SUMMARY

1. A new enzyme, cytochrome *c* peroxidase, which specifically catalyzes the reaction between cytochrome *c* and hydrogen peroxide is reported and the method for isolating it from bakers' yeast is described.

2. The spectrophotometric test for this peroxidase is described in detail.

3. The absorption spectra of both the oxidized and reduced forms of the enzyme preparation are given.

4. A comparison between the activities of cytochrome *c* peroxidase and horseradish peroxidase toward both cytochrome *c* and pyrogallol is made and it is demonstrated that these enzymes are two different entities.

5. This enzyme is inactivated by heating to 55° and its activity is completely inhibited by small amounts of cyanide and to a much lesser extent by phosphates and chlorides.

6. The purest preparations thus far obtained have a hemin content of 0.30 per cent. A modification of the method of Kuhn, Hand, and Florkin for the determination of hemin is described.

7. There is no apparent relationship between the copper content and the activity of the enzyme preparation nor is the enzyme inhibited by diethyl dithiocarbamate, which is specific toward copper.

8. A comparison between the activities of cytochrome *c* peroxidase and catalase shows that the peroxidase is as efficient as the most active catalase preparation in the utilization of hydrogen peroxide.

9. Yeast is shown to contain about 250 times as much cytochrome *c* peroxidase as catalase.

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SULFHYDRYL GROUPS OF SERUM ALBUMIN, SERUM, AND MILK

Sirs:

The maximal number of sulfhydryl groups in many proteins is revealed in guanidine halide solutions.[1,2] These groups have been estimated by the porphyrindin-nitroprusside method.[3] The porphyrindin is standardized against cysteine before, during, and after the titration of the protein. Recently, Anson has confirmed our values for egg albumin in guanidine hydrochloride, using various oxidants as titrating agents.[4] The agreement is satisfactory and suggests that sulfhydryl groups in proteins can be readily estimated when nitroprusside is used as an outside indicator.

Serum albumin denatured by heat or by urea gives no tests characteristic for sulfhydryl.[5] In solutions of guanidine hydrochloride, however, this protein gives a strong nitroprusside reaction. This striking difference in the relative effects of urea and of guanidine hydrochloride confirms data already given for other proteins.[1,3] The amount of —SH groups liberated in a 3 per cent solution of purified beef serum albumin in guanidine hydrochloride was estimated by the method described[3] and the results expressed in terms of cysteine per 100 gm. of protein. The cysteine revealed was 0.34 per cent in 8 to 16 M guanidine hydrochloride. Below 8 M guanidine less cysteine was progressively revealed, and at 1 M the reaction for —SH was negative. The minimal weight calculated from these data is 35,300.

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When solutions of the protein in concentrated guanidine hydrochloride were allowed to stand exposed to the air for 2 hours, no diminution in the amount of —SH groups could be observed. This is similar to results of earlier work with egg albumin,[1] and indicates the absence of appreciable amounts of heavy metals from our preparations. Neutralized cysteine hydrochloride added to a solution of the native serum albumin in water could be estimated within 5 per cent. Rosenthal and Voegtlin have shown that the autoxidation of denatured egg albumin is negligible even in the presence of iron.[6]

Serum or milk treated with 8 to 16 M guanidine hydrochloride gives a positive nitroprusside reaction. The cysteine revealed, calculated as mg. per mg. of protein nitrogen, is for sera as follows: rabbit 0.010, rat 0.004, guinea pig 0.009, and dog 0.010. Cow's milk becomes nearly transparent when treated with guanidine hydrochloride and the cysteine revealed amounts to 0.008 mg. per mg. of protein nitrogen. Each fraction of the serum proteins shows sulfhydryl groups; in milk the sulfhydryl reaction is found in the albumin and globulin fractions and not in casein. Studies of the sera of tumor-bearing animals will be reported elsewhere.

National Cancer Institute
National Institute of Health
Bethesda, Maryland

JESSE P. GREENSTEIN

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OXIDATION OF GLYCOGEN BY BRAIN SUSPENSIONS

Sirs:

If glycogen is added to a suspension of rat brain, prepared by homogenization in the apparatus of Potter and Elvehjem,[1] a considerable increase in the rate of oxygen uptake occurs. The mechanism of glycogen oxidation is evidently different from that of glucose, since the effect of glycogen and glucose added together is, during the first 20 minutes of readings, almost exactly equal to the sum of their effects when added separately. Nearly pure gray matter behaves similarly to whole brain. The concentrations of glucose (0.2 per cent) and of glycogen (0.7 per cent) used gave maximal effects when added separately; increases in the concentration of either alone had no effect.

Pyruvate increases the rate of respiration more than does glucose, but glucose and pyruvate together do not give any additive effect, while pyruvate and glycogen together give a completely additive effect during the first 20 minutes. Hexose diphosphate has no effect in the presence or absence of glucose or glycogen other than to cause a slight inhibition.

The results obtained depend upon the method of preparation of the tissue. With slices of gray matter in Ringer-phosphate medium the addition of glucose causes a very large increase in the respiration rate, while glycogen addition has no effect. Whole brain suspensions tested in NaCl-phosphate medium, isotonic with serum, show both the glucose and the glycogen effects. If the tissue, whole brain or gray matter, is homogenized and tested in hypotonic medium, the respiration without addition is quite low, glucose has very little effect, and glycogen has an increased effect. Evidently cytolysis allows the glycogen to gain access to the enzymes which attack it while disrupting the mechanisms which deal with glucose. In all cases, after 30 to 45 minutes, the respiration rate in the presence of glycogen, or glycogen plus glucose, falls off more than in the presence of glucose alone.

[1] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).

The table gives the results of typical experiments illustrating the above statements.

Preparation	Time	O_2 uptake without substrate	Extra O_2 uptake in presence of: Glucose	Glycogen	Glucose + glycogen	Pyruvate	Pyruvate + glucose	Pyruvate + glycogen	Pyruvate + glucose + glycogen
	min.	*c.mm.*							
Mixed whole brain, 300 mg. fresh weight									
Homogenized in hypotonic solution and run in isotonic solution	0–20	94	43	33	76	78	77	101	101
	90–120	50	61	16	35	72	72	54	46
Homogenized and run in isotonic solution	0–20	119	61	34	88				
Homogenized and run in hypotonic solution	0–20	55	9	52	64				
Gray matter, 300 mg. fresh weight									
Homogenized and run in hypotonic solution	0–20	86	5	69	73				
Gray matter, 22 mg. final dry weight (approximately 150 mg. fresh weight)									
Slices in Ringer-phosphate	0–20	42	66	1	67				
	90–120	14	139	−1	118				

The observations here reported indicate definitely that at least two mechanisms of carbohydrate oxidation occur in brain, one applying to glycogen and one to glucose. The possibility that other tissues behave similarly to brain in this respect will be tested shortly.

Institute of the Pennsylvania Hospital
Philadelphia

K. A. C. Elliott
B. Libet

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THE SOURCE OF CARBON FOR UREA FORMATION*

Sirs:

According to the prevailing concept urea is formed by mammals through the ornithine-arginine cycle.[1] Carbon dioxide has generally been assumed to supply the carbon.

The reaction has now been investigated with the aid of the stable isotope C^{13}. Washed liver slices (dry weight 1.5 gm.) were shaken in 70 cc. of buffer with added NH_4Cl, ornithine, and glucose, as described by Krebs and Henseleit.[1] The bicarbonate of the buffer contained 17.2 atom per cent C^{13} excess.[2] After 2 hours shaking with oxygen at 37° the supernatant fluid was deproteinized with alcohol containing 3 per cent acetic acid. The CO_2 thereby evolved contained 12.7 atom per cent C^{13} excess. Normal CO_2 was then passed through the solution to remove any trace of marked carbon dioxide which still might have been present. The solution was taken to dryness, and the residue extracted with alcohol. Evaporation and extraction with alcohol were repeated three times and the residue finally dissolved in 10 cc. of water. This solution contained no detectable amounts of carbon dioxide.

The urea present in the solution was decomposed with urease and the CO_2 evolved was absorbed in barium hydroxide solution. The resulting 18 mg. of $BaCO_3$ contained 6.3 atom per cent C^{13} excess; about half of the carbon of the urea must therefore have been derived from the labeled bicarbonate of the buffer. The remainder might have been derived from the CO_2 originating from the tissue respiration. Neither the total proteins of the tissue slices nor the carbon of the amidine group of the arginine

* This investigation was supported by a grant from the Rockefeller Foundation and by a grant from the Friedsam Fund, donated to the Division of Child Neurology, Neurological Institute, New York.

[1] Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).

[2] The authors are indebted to Dr. H. C. Urey for the valuable gift of the isotope.

isolated from the proteins contained any marked carbon above the error of the analytical procedure. The analyses of the isotope concentrations were carried out with a mass spectrometer.

Department of Biochemistry
College of Physicians and Surgeons
Columbia University
New York

D. RITTENBERG

Department of Biochemistry
New York State Psychiatric Institute and Hospital, and the Department of Neurology, Columbia University
New York

HEINRICH WAELSCH

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A CONSTITUENT OF RAW EGG WHITE CAPABLE OF INACTIVATING BIOTIN IN VITRO

Sirs:

Recently[1] the tissues of chicks on a diet causing egg white injury were shown to be deficient in biotin (vitamin H) despite the abundance of this vitamin in the diet. The injury caused by egg white was concluded to be due to the action of egg white in making biotin unavailable.

It occurred to one of us (R. E. E.) that an inactivation of biotin by egg white *in vitro* might be demonstrated. This was found to be the case and the biotin-inactivating capacity of commercial egg albumin was found to be nearly the same whether the biotin preparation was pure (Kögl) or a very crude concentrate. In the case of pure biotin 2.2 γ were inactivated by 1 gm. of egg albumin, while in a crude yeast extract 1.8 γ were inactivated by the same amount.

The biotin test method was that developed in this laboratory[2] and the effect of the "egg albumin" was observed simply by introducing it into the sterile biotin solution previous to the test, and then carrying out the test without heat sterilization. An aliquot of the same solution sterilized with heat (steaming) served as a positive control.

The constituent of raw egg white responsible for thus rendering the biotin unavailable for yeast growth stimulation (without any toxic action) has been purified as follows: 8 liters of acetone were added to 2 liters of fresh egg white and the coagulum was washed with 1 liter of water. It was then thoroughly suspended and washed with 2 liters of 1 per cent salt solution and the filtrate retained. The filtrate was five-sixths saturated with ammonium sulfate and filtered, the precipitate being discarded. The active

[1] Eakin, R. E., McKinley, W. A., and Williams, R. J., *Science*, **92**, 224 (1940).

[2] Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, **62**, 175 (1940).

fraction was then salted-out by complete saturation with ammonium sulfate. After filtration the material was dissolved in a small volume of water and exhaustively dialyzed in a cellophane bag against tap water.

1 part of biotin was inactivated by 125 parts of this preparation. It represented 1000-fold concentration (and approximately 35 per cent yield) of the inactivating substance, calculated on the basis of the amount in the fresh egg white.

The union between the biotin and the unheated protein is such that biotin is not released by dialysis in the pH range 2 to 10.5. Steam sterilization completely releases the biotin. The ability of this substance to take up and release biotin specifically and quantitatively suggests its possible use as a tool in the purification of biotin.

The action of this substance in rendering biotin unavailable to yeast makes it highly probable that it is the constituent in raw egg white which produces "egg white injury" in animals. Its further purification and physiological significance are being investigated.

Department of Chemistry
University of Texas
Austin

Robert E. Eakin
Esmond E. Snell
Roger J. Williams

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BACTERICIDAL FRACTIONS FROM AN AEROBIC SPORULATING BACILLUS

Sirs:

The present communication is intended to describe simplified procedures for purification of the fractions obtained from the bactericidal agent produced by an aerobic sporulating bacillus.[1, 2] A revision of the earlier conclusions regarding the chemical nature and biological activity of certain of the fractions is also presented.

Gramicidin can be obtained directly by extracting the crude alcohol-soluble material repeatedly with a mixture of equal volumes of acetone and ether. The extracts are evaporated and the portion of the residue which is soluble in acetone gives crystalline gramicidin when dissolved in warm acetone and cooled.

That portion of the crude material (approximately 85 per cent) which remains insoluble in acetone-ether is dissolved in about 4 times its weight of boiling absolute alcohol and there is added alcoholic hydrogen chloride corresponding to 0.1 of a mole per liter. A precipitate forms as the solution cools. This precipitate, when recrystallized several times from absolute methanol with the use of small amounts of hydrogen chloride, separates in clusters of microscopic needles, melting at 237–239° (uncorrected; Berl-Kullmann copper block) with decomposition and showing $[\alpha]_D^{25} = -102°$ (95 per cent alcohol solution; concentration 1 per cent). Analysis reveals C 59.4, H 6.8, N 13.5, Cl 2.7. This substance was described earlier under the name *graminic acid* before its low chlorine content was recognized; it will be referred to hereafter as *tyrocidine hydrochloride*. It is the salt of a weak base (pK′ = about 8.5) and tends to crystallize without its full complement of hydrochloric acid. This tendency may be responsible for small variations observed in the analytical properties, and for the recovery of a fraction called in the earlier report "gramidinic acid" but now considered to be a mixture of tyrocidine and its salt.

[1] Dubos, R. J., and Cattaneo, C., *J. Exp. Med.*, **70**, 249 (1939).

[2] Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, **132**, 791 (1940).

Gramicidin will continue to be used as the name of the crystalline neutral substance described before.[2] It is proposed here to apply the name *tyrothricin* to the bactericidal agent prepared as an alcohol-soluble, water-insoluble material and containing both gramicidin and tyrocidine. This name is derived from the word *Tyrothrix*, a generic name first used by Duclaux to designate sporulating aerobic bacterial species, several of which have since been found to exhibit antagonistic activity toward other microorganisms.[3]

It may be noted here that tyrothricin and tyrocidine hydrochloride are now known to show under suitable conditions a marked degree of bactericidal activity *in vitro* against Gram-negative as well as Gram-positive microorganisms. Gramicidin, however, under the same conditions shows a high degree of specificity in attacking only the Gram-positive organisms. Furthermore, it can now be stated that tyrocidine hydrochloride in amounts of 50 to 100 γ shows definite protective action in mice infected intraperitoneally with pneumococci. As stated previously, gramicidin protects mice at a much lower level, *viz.* 1 to 5 γ, under the same conditions.

Hospital of The Rockefeller Institute for Medical Research
New York

Rollin D. Hotchkiss
René J. Dubos

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[3] Much, H., *Med. Klin.*, **20**, 347 (1924). Rosenthal, L., *Compt. rend. Soc. biol.*, **92**, 78 (1925).

THE RÔLE OF CARBON DIOXIDE IN THE SYNTHESIS OF UREA IN RAT LIVER SLICES*

Sirs:

The rate of synthesis of urea in mammalian liver slices increases rapidly with increasing concentrations of the bicarbonate-CO_2 buffer of the medium.[1] This specific effect has been explained on the assumption that the first stage in the synthesis of urea is the formation of δ-carbaminoornithine,[2] the carbon of the urea molecule being derived entirely, therefore, from the bicarbonate of the medium. We have tested this mechanism by studying the synthesis of urea by rat liver slices, in a medium containing radioactive bicarbonate. The pertinent data are listed in the table.

100 ml. of Krebs saline-bicarbonate (containing C_{11}); 95 per cent O_2-5 per cent CO_2; pH 7.4; 200 mg. per cent of ornithine; 20 mg. per cent of NH_4Cl; 200 mg. per cent of pyruvate; 45 minutes; 40°.

Experiment No.	Liver slices, wet weight	Urea formed	Activity per mg. carbon*	
			Urea	Added $NaHCO_3$
	gm.	*mg.*		
1	1.57	8.8	0.09	0.22
2	1.42	6.8	0.08	0.21
3	1.05	3.8	0.02	0.05

* Divisions per second (Lauritsen electroscope); corrected for decay, so that the values are comparable.

At the end of the experimental period the liver slices were removed and the solution acidified with 2 volumes of glacial acetic acid. 50 mg. of urea were added to act as a carrier and the urea precipitated by a solution of xanthydrol in methyl alcohol. The

* This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[1] Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).

[2] Krebs, H. A., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, **3**, 247 (1934).

precipitate was centrifuged off, washed with dilute acetic acid, methyl alcohol, dried *in vacuo*, weighed, and its activity measured by a Lauritsen electroscope. The dixanthylurea melted at 266° and showed no depression of the melting point when mixed with a control sample.

The data show without question that the bicarbonate of the medium is utilized in the synthesis of urea. The fact that the isotopic concentration of the urea is less than half that of the bicarbonate of the medium can probably be entirely ascribed to dilution with metabolic CO_2 and to exchange with the CO_2 of the gaseous phase of the experimental vessel.

Department of Biochemistry
University of Chicago
Chicago

E. A. Evans, Jr.

Department of Physics
University of Chicago
Chicago

Louis Slotin

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THE ISOLATION OF ETIOALLOCHOLANOL-3(β)-17-ONE (ISOANDROSTERONE) FROM NORMAL AND PATHOLOGICAL HUMAN URINES*

Sirs:

Etioallocholanol-3(β)-17-one has been previously isolated from the urine of a woman with adrenal cortical pathology[1] and from the urine of adult male guinea pigs injected with testosterone propionate.[2] We wish to report this steroid as a constituent of the urine of normal women. It also occurs in the urines of patients with cancer. Its presence in the urine of normal men is indicated.

The ketonic material of ether extracts of acid-hydrolyzed urines was separated into alcoholic and non-alcoholic fractions by half esterification with succinic anhydride. The digitonin-precipitable hydroxy ketones were purified by the preparation of crystalline semicarbazones and subsequent hydrolysis.

From 146 liters of urine of normal women, there were obtained 30 mg. of a digitonin-precipitable hydroxy ketone melting at 165°. Two recrystallizations from ethanol and high vacuum distillation yielded 15 mg. of a crystalline sublimate ($[\alpha]_D^{20} = +78°$, 0.37 per cent in ethanol). Recrystallization from methanol gave 11 mg. melting at 168–169°; a mixed melting point with authentic isoandrosterone (m.p. 170–171°) was 169–171°. An additional 10 mg., obtained from the mother liquors, melted at 162–164°; the benzoate melted at 213–215° and did not depress the melting point of isoandrosterone benzoate (m.p. 219–220°).

Similarly, the urine of female patients with cancer yielded 5 mg., melting at 161–163°. The benzoate, m.p. 210–211°, gave no depression with isoandrosterone benzoate. 10 mg., m.p. 165°, were obtained from the urine of male patients with cancer. The

* Aided by grants (administered by G. Pincus) from the Dazian Foundation for Medical Research and G. D. Searle and Company. Works Progress Administration Project 65-1-14-2949.

[1] Butler, G. C., and Marrian, G. F., *J. Biol. Chem.*, **124**, 237 (1938).

[2] Dorfman, R. I., and Fish, W. R., *J. Biol. Chem.*, **135**, 349 (1940).

benzoate, on three recrystallizations from ethanol, melted at 215–217°; its mixed melting point with isoandrosterone benzoate was 218–219°. Analysis, $C_{26}H_{34}O_3$, calculated, C 79.14, H 8.69; found, C 78.80, H 8.55.

The digitonin-precipitable hydroxy ketonic fraction of normal male urine yielded a crystalline product, melting sharply at 156.5–157.5° ($[\alpha]_D^{20} = +50°$, 1.09 per cent in ethanol). Analysis, $C_{19}H_{28}O_2$, calculated, C 79.11, H 9.79; $C_{19}H_{30}O_2$, calculated, C 78.57, H 10.41; found C 78.83, H 9.88. A mixture of dehydroisoandrosterone and isoandrosterone is suspected.

Androsterone and two of its isomers, etioallocholanol-3(β)-17-one and etiocholanol-3(α)-17-one, are present in human urines. The remaining stereoisomer of androsterone, etiocholanol-3(β)-17-one, if at all present, would be found in the digitonin-precipitable hydroxy ketonic fraction. In any event, a current assumption[3] that dehydroisoandrosterone is the sole constituent of this fraction must be revised.

Physiological Laboratories
Clark University
Worcester

W. H. Pearlman

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[3] Hoskins, W., and Webster, B., *Proc. Soc. Exp. Biol. and Med.*, **43**, 604 (1940).

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